The botanical origin, physicochemical properties and antibacterial activity of selected West Coast honeys

by

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Abstract

Honey is a complex natural product made by honey bees. In recent years consumer preference in the market has shifted towards honeys with distinct characteristics such as unique floral origin (i.e. monoflorals) and potential health benefits. The specific physical and chemical properties that make every honey unique are primarily influenced by the distinct nectar composition of the plants that were visited by the bees. The Cape Floristic Region (CFR) is characterised by high plant species diversity and exceptionally high species endemism. With the close association between botanical origin and honey properties in mind, this study aimed to identify novel honeys produced from fynbos plants along the West Coast of South Africa that could be marketed as an exclusive, niche product to higher tier local and international consumers. The botanical composition of honeys was investigated by generating the first pollen library for the West Coast and using melissopalynology to identify honey floral components and monofloral varieties. Honey bees preferred and utilised similar floral sources across space and time, with differences in botanical origin between years ascribed to differences in floral availability, due to changes in rainfall. Sandbos (*Aspalathus spinescens* form A) was the most abundant monofloral honey produced. The physicochemical composition of honey also varied over space and time, as well as with honey age. However, the majority of honeys produced along the West Coast were of good quality and complied with local as well as international regulations – fresh and after 12-months in storage. Hydrogen peroxide was responsible for the antibacterial activity of the West Coast honeys, which was confirmed using a phenol equivalence agar well diffusion assay. Almost 70% of honeys were potentially therapeutically useful, although the variation in antibacterial activity of specific monofloral honey varieties was quite high. Unfortunately, the sample sizes of different honey varieties were limited due to the severe drought conditions experienced between 2015 and 2017, making it difficult to give definitive recommendations on honey properties to be used for value-added marketing. Climate change scenarios predict that the West Coast will become warmer and drier in the future and environmental fluctuations such as changes in rainfall and temperature that affect nectar availability will also greatly influence honey production. The beekeeping industry should focus on optimising the honey yield from drought-tolerant plant species such as sandbos, through the identification and preservation of sites where these plants occur, or through cultivation. This approach of botanical, physicochemical and antibacterial characterisation of honeys produced from indigenous flora could be extended over larger areas of the CFR with the help of beekeepers and citizen science initiatives. Research characterising the unique honeys produced from indigenous vegetation must continue, increasing honey's value on local as well as international honey markets and in turn boosting the beekeeping industry of South Africa.

Opsomming

Heuning is 'n komplekse natuurlike produk wat deur heuningbye vervaardig word. In die laaste jare het verbruikersvoorkeur in die mark verskuif na heuning met spesifieke eienskappe, soos unieke plantoorsprong (d.w.s. monoflorale heuning) en potensiële gesondheidsvoordele. Die spesifieke fisiese en chemiese eienskappe wat elke heuning uniek maak, word hoofsaaklik bepaal deur die nektarsamestelling van die plante wat deur bye besoek word. Die Kaapse Floristiese Streek (KFR) word gekenmerk deur 'n groot verskeidenheid plantspesies en baie hoë spesie-endemisme. Met die noue verband tussen heuning-eienskappe en botaniese oorsprong in gedagte, was die doel van hierdie studie om nuwe heuningsoorte, afkomstig van fynbosplante langs die Weskus van Suid-Afrika, te identifiseer wat as 'n eksklusiewe nisproduk aan plaaslike en internasionale verbruikers bemark kan word. Die botaniese samestelling van heuning is ondersoek deur die eerste stuifmeelbiblioteek vir die Weskus saam te stel en melissopalinologie te gebruik om heuning-blomkomponente en monoflorale variëteite te identifiseer. Heuningbye verkies en benut soortgelyke blombronne in ruimte en tyd en verskille in botaniese oorsprong tussen jare word toegeskryf aan verskille in blombeskikbaarheid weens wisselende reënval. Sandbos (*Aspalathus spinescens* vorm A) was die volopste monoflorale heuning wat geproduseer is. Die fisies-chemiese samestelling van heuning het ook met ruimte en tyd gewissel, asook met heuningouderdom. Die meerderheid heunings was egter van goeie gehalte en het aan plaaslike sowel as internasionale standaarde voldoen – vars en na 12 maande stoortyd. Waterstofperoksied was verantwoordelik vir die antibakteriese aktiwiteit van die heunings, wat bevestig is met behulp van 'n fenol-ekwivalensie agar-diffusietoets. Byna 70% van die heuning was potensieel terapeuties bruikbaar, hoewel die variasie in antibakteriese aktiwiteit van spesifieke monoflorale heuningvariëteite redelik groot was. Ongelukkig was die monstergroottes van verskillende heuningvariëteite beperk vanweë die ernstige droogtetoestande wat tussen 2015 en 2017 ervaar is, wat dit moeilik gemaak het om definitiewe aanbevelings te maak oor heuningeienskappe wat vir waardetoevoegingsbemarking gebruik kan word. Scenario's vir klimaatsverandering voorspel dat die Weskus in die toekoms warmer en droër sal word. Fluktuasies in die omgewing, soos veranderinge in reënval wat die beskikbaarheid van nektar beïnvloed sal ook heuningproduksie beïnvloed. Die byebedryf moet fokus op die optimering van heuningopbrengs van droogtebestande plantspesies soos sandbos, deur die identifisering en bewaring van areas waar hierdie plante voorkom, of deur aanplanting. Hierdie benadering van botaniese, fisies-chemiese en antibakteriese karakterisering van heuning, kan toegepas word oor groter dele van die KFR met behulp van inisiatiewe vir burgerwetenskap. Navorsing wat unieke heunings karakteriseer wat vanaf inheemse plantegroei geproduseer word, moet voortduur om die waarde van heuning op plaaslike sowel as internasionale heuningmarkte te verhoog en sodoende byeboerdery in Suid-Afrika te bevorder.

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Chapter 1: General introduction

Humans have lived in close contact with honey bees since prehistoric times (Roffet-Salque et al. 2015). Apiculture has played a significant role in many cultures over human history, where honey and other honey bee products such as wax and propolis have been used as a food source and for medicinal purposes for thousands of years. In more recent history, the western honey bee (*Apis mellifera* L., hereafter referred to as honey bees) has been providing indispensable pollination services that are not only vital in supporting many terrestrial ecosystems, but also in maintaining global agricultural crop production (Klein et al. 2007; Breeze et al. 2014).

Honey bees are cavity-nesting, eusocial hymenopterans in the family Apidae (subfamily: Apinae) that exist in caste-based colonies consisting of a single queen, brood, thousands of female workers and seasonal male drones. Worker bees perform tasks both inside and outside the nest, graduating between duties based on their age (temporal polyethism) and influenced by the current needs of the colony (Ribbands 1952; Winston and Fergusson 1985). Thereafter the worker bees start to perform out-colony tasks, which include the collection of water and resin as well as pollen and nectar from flowering plants (Abou-Shaara 2014). Scouting foragers returning to the colony relay information about the location of worthwhile food sources to other foragers, who in turn set out to collect the nectar and pollen based on the scout bees' dance (Anderson and Ratnieks 1999; Dornhaus and Chittka 2004). Through the process of collecting the nectar and pollen for their colony needs, honey bees inadvertently play a significant ecological role as pollinators of flowering plants.

Approximately 60% of honey bee foragers collect nectar, 25% collect pollen and 15% collect both pollen and nectar on foraging trips (Parker 1926; Winston 1987). Honey bees usually travel between 0.6 km and 1.5 km from the colony to obtain these resources (Beekman and Ratnieks 2000; Beekman et al. 2004; Danner et al. 2016), but foragers have been recorded flying up to 13 km over desert areas to reach agricultural crops (Crane 1975a). Honey bees prefer a mixture of sugars above any one particular sugar and this, as well as the concentration of sugar in nectar, are factors that might influence choice of forage (Maurizio 1975a). When nectar foragers return to the colony their nectar load is transferred from the gatherer's honey-stomach to a receiving in-colony worker through a back-and-forth regurgitation process, after which the sugary liquid (at this point enriched with enzymes added by the bees) is finally deposited into comb cells. The honey bees fan their wings to evaporate excess water from the cells and the cells are capped with wax after the liquid is sufficiently thickened. Honey is the final concentrated product resulting from this process and is stored as a carbohydrate food source in the colony to satisfy immediate energy requirements as well as to provide sustenance in times of nectar scarcity. Pollen foragers gather and transport pollen in specialised pollen baskets (corbiculae) located on the tibia of honey bee hind legs, after which it is mixed with nectar and glandular secretions and also stored in comb cells inside the colony to serve as a protein source for feeding larvae (Winston 1987). A single honey bee colony in South Africa requires about 30 to 60 kg of honey per year to survive (Johannsmeier 2001a).

In ecosystems where *A. mellifera* subspecies are indigenous (Africa, Europe and Asia), they have evolved alongside native flora as generalist pollinators who visit and service a variety of flowering plant taxa. Several characteristics of honey bees distinguish them from other insect pollinators e.g. their high flower constancy (up to 97% intraspecific pollen transfer; Tribe and Allsopp 2001a) and their potential to have a high numerical abundance in the landscape. Colony densities vary greatly between geographic locations because of differences in environmental factors such as nesting site and food source availability, which is mostly influenced by climate and human-mediated landscape change (Jaffé et al. 2010; Hinson et al. 2015). Studies using drone genotyping techniques to determine colony densities of wild or feral honey bees have estimated 0.1 to 1.5 colonies per km^2 in South East Australia (Hinson et al. 2015), 3 colonies per km^2 in Europe (Jaffé et al. 2010) and approximately 10 colonies per km^2 in South Africa, in good honey bee areas (Moritz et al. 2008). In turn, each of these honey bee colonies can consist of 10,000 to 60,000 individuals of which up to 40% are active foragers and thus potential pollinators (Tribe and Allsopp 2001b).

Nevertheless, this seemingly high abundance of honey bees in the landscape, together with other insect pollinators, varies greatly in time and space and even at their highest densities in the wild are only a fraction of the amount of pollinators needed to meet the unnaturally high demand for agricultural crop pollination. Globally the total economic value of pollination was estimated to be €153 billion in 2005 (Gallai et al. 2009) and approximately one third of agricultural crops globally depend on insect pollination (Klein et al. 2007). Large-scale commercial crops usually occur in vast monoculture stands, which result in extensive degradation of natural habitat and inadequate vegetation to sustain wild pollinators. Additionally, pollinators worldwide are also under threat by increased pesticide use, the spread of pests and diseases, anthropogenic climate change and various other factors associated with the present global decline in biodiversity (Melin et al. 2014). It is therefore not surprising that the honey bee, an easily managed and numerically abundant generalist pollinator, have become actively managed by beekeepers worldwide to satisfy this high pollination demand.

The area of insect pollinated crops has increased globally by between 70 and 100% from 1961 to 2006 (Aizen et al. 2008) and depending on the crop, approximately 10 to 1000 honey bee colonies are needed per square km to ensure optimal production (Breeze et al. 2014). Except for small-scale beekeepers who mainly keep their bees for the production of honey and other bee related products, the majority of managed honey bees worldwide are kept primarily for the purpose of providing the vast amounts of colonies needed for fruit, nut and vegetable pollination. Although this is not the case globally, commercial beekeepers in the USA generate a significant income renting out beehives - equal to and surpassing the income from honey sales (Morse and Calderone 2000; Phillips 2014). However, renting out beehives for pollination services does not always contribute to honey production, and only certain crops throughout the year have the added benefit of supplying beekeepers with forage to help sustain their beekeeping operations in what would otherwise be resource scarce times. Thus, to support their honey bees during periods when no honey producing agricultural crops are flowering, beekeepers must either feed their honey bees or rely on flowering plants of naturally occurring indigenous or alien vegetation to sustain their numerous bee colonies. Additionally, honey bees require a diverse diet to maintain colony health, which provides another good reason to rotate colonies between usually monoculture agricultural crops and generally more diverse natural vegetation (Brodschneider and Crailsheim 2010).

Natural plant community compositions vary spatially and temporally, producing a unique suite of forage plants available to honey bees at different geographic locations, in different climates and between seasons (Gaston 2000; Soininen 2010). The quantity and quality of these forage resources available in the field directly influence honey bee activity patterns, which in turn affect the botanical origin (discussed in Chapter 2), quantity and quality of honey in the hive (Butler 1945; White et al. 1962; Abou-Shaara 2014). For example, the abundance of flowering plants (together with environmental factors such as humidity) dictates the nectar flow available to foragers and subsequently influences the amount of honey being produced (Porter 1987; McNally and Schneider 1992). In turn, each species of flowering plant also has its own unique nectar composition with regards to sugar spectrum and phenolic compounds, which influence honey composition (Moniruzzaman et al. 2014). Therefore, the available floral resources affect the rates of honey production and define its botanical composition that directly determines the product’s physical and chemical properties (El-Sohaimy et al. 2015; Warui et al. 2019). Physicochemical properties of honey (discussed in Chapter 3) have fixed internationally acceptable values, which help to protect product integrity by allowing the detection of product adulterations by honey producers (Anklam 1998; Bogdanov 2009). The unique composition of honey is also directly related to sensory characteristics such as honey taste, colour and odour as well as other properties including antioxidants and antibacterial activity of honey (Bogdanov et al. 2004; Kwakman et al. 2010; Irish et al. 2011; Wanjai et al. 2012).

The antibacterial activity of honey (discussed in Chapter 4) has been well-documented throughout human history (Lee et al. 2011; Israili 2014; Miguel et al. 2017). The treatment of ailments with honey has recently gained increased recognition in the health care sector (Molan and Betts 2004; Mandal and Mandal 2011; Israili 2014; Miguel et al. 2017) and honeys that exhibit superior antibacterial properties are valued in both the pharmaceutical sector as well as with private consumers who prefer a more natural approach to healthcare. The characteristics of a good antibacterial honey are low pH, high osmolarity and especially the production of hydrogen peroxide (H_2O_2) by the enzyme glucose oxidase (Irish et al. 2011; Kwakman and Zaat 2012; Bucekova et al. 2019). The specific physicochemical composition of honey with regards to sugar content, acidity, enzymatic activity and phytochemical compounds can also affect this antibacterial potential (Mandal and Mandal 2011). Thus, antibacterial activity of honey varies greatly between honeys from different floral sources (Taormina et al. 2001). One example is manuka honey produced in New Zealand by honey bees foraging on the manuka tree, *Leptospermum scoparium* J.R. Forst. & G. Forst. Manuka is well-known for its unique antibacterial properties attributed to a specific compound, methylglyoxal (MGO) originating from the flower nectar (Allen et al. 1991; Kato et al. 2014). The activity level resulting from this compound equates to what is called the honey's Unique Manuka Factor (UMF) for which it is world-renowned. This is a value assigned to each batch of honey based on the non-peroxide antibacterial activity, which uniquely increases as the honey ages. In the majority of other honeys that usually exhibit antibacterial activity due to hydrogen peroxide content, the activity typically decreases with honey age (Irish et al. 2011; Chen et al. 2012).

According to the Food and Agriculture Organization of the United Nations (FAOSTAT 2019) approximately 1.86 million tonnes of honey were commercially produced worldwide in 2017, with China, Turkey and Argentina being the top three honey producing countries contributing 29.18%, 6.15% and 4.1% of the global production, respectively. An estimated 198,959 tonnes of honey were produced in Africa (10.7% of the global crop) of which Ethiopia is the biggest contributor at almost 50,000 tonnes. In 2017 South Africa produced an estimated 1088 tonnes of honey, which is 0.55% of honey produced in Africa and less than 0.1% of honey produced globally (FAOSTAT 2019). This is considerably less than the estimated production in South Africa in 2007, which was around 2250 tonnes (NAMC 2008) and about 25% to 50% of what was produced in the country in the 1970's (Allsopp and Cherry 2004). Additionally, the reported ca. 30% decline in honey bee colonies from 2009 to 2010 and 46% decline from 2010 to 2011 also suggest that South African beekeeping is indeed suffering under the same pressures experienced by this industry globally (Pirk et al. 2014).

A dwindling honey bee industry not only puts South Africa at risk of not meeting the country's pollination demands (particularly the Western Cape with a deciduous fruit industry worth ZAR 9800 million per year; Melin et al. (2014)), but also impacts on honey production. In 2007 there was already a honey shortfall of about 1000 tonnes, which had to be imported to meet the increasing national honey demand (NAMC 2008). Out of all the Southern African Development Community (SADC) countries South Africa is the biggest importer of honey, amounting to 73% of all SADC honey imports in 2005 and equalling US$ 1.2 million. From this US$ 540,000 was spent on imports from China and US$ 480,000 from Argentina in the same year (AusAID 2016). In order to set South African honeys apart from these blended bulk imports, a high value niche honey must be identified that will appeal to elite honey consumers, locally and abroad, that can sustain the beekeeping industry in the face of adversity.

In recent years, a growing trend in consumer preference has developed towards organic and healthy produce, alongside the increasing popularity of food products labelled as local or from specific geographic regions (Rozin et al. 2004; Bond et al. 2008; Hu et al. 2011). This is also true for the honey market, with raw and unprocessed honeys produced from indigenous and endemic flora fetching higher prices with consumers (Unnevehr and Gouzou 1998; Batt and Liu 2012; De la Guardia and Illueca 2013). Honeys with unique properties that are predominantly from a single floral origin (called monofloral honeys) are also increasing in popularity. As early as 1975, Eva Crane reported that honeys from a specific source of origin (geographic as well as botanical) are becoming more sought after than blended, standardised honeys with ordinary characteristics (Crane 1975b; Crane 1975c). Depending on the availability and the specific characteristics of certain monofloral varieties, prices can range anywhere between €4 per kg for common honeys and at least €15 per kg for unique varieties with proven medicinal value (CBI Market Intelligence 2015). However, despite these new trends and opportunities to enter the global honey market with niche products, limited research has been done on South African honeys and the beekeeping industry is not making adequate use of the potential to market honey products produced from unique indigenous vegetation.

South Africa is famous for its high diversity of biota and the Western Cape Province, especially, has a large variety of plant species. The Cape honey bee, *Apis mellifera capensis* Eschscholtz, is native to the Western Cape Province and the Cape Floristic Region (CFR) biodiversity hot spot, the smallest of the six recognized floral kingdoms of the world (Good 1947) hosting more than 9000 plant species, of which 70-80% are endemic (Manning and Goldblatt 2012). The vegetation in the CFR is locally called "fynbos", although the fynbos biome is actually a Mediterranean ecogeographic region located within the CFR. The fynbos biome is characterised by the presence of an ericoid component (fine-leaf shrub species from e.g. the Ericaceae, Fabaceae and Asteraceae families), a proteoid component of species in the family Proteaceae and a restioid component of species in the Restionaceae family (Bergh et al. 2014). The biome is further subdivided into three major vegetation complexes, i.e. fynbos, renosterveld and strandveld – each in turn divided into many different vegetation units that are characterised by specific soil types, climatic conditions and unique endemic plant species found nowhere else on earth (Rebelo et al. 2006; Bergh et al. 2014).

With the close association between botanical origin and honey properties discussed above, it is reasonable to expect that honey produced by honey bees from the CFR region within the Western Cape might also have their own distinctive characteristics that reflect the area's unique floral bouquet. Unfortunately, the properties of "fynbos" honey in South Africa have not been extensively studied. They are restricted to one study on the botanical origin of honey (Johannsmeier 2001b), one study on the physicochemical properties of honey (Anderson and Perold 1964) and four studies on the potential antimicrobial properties of honey (Theunissen et al. 2001; Basson and Grobler 2008; Manyi-Loh et al. 2013; Khan et al. 2014). This study will thus endeavour to identify a novel honey that appeals to a higher tier local and international consumer by using a multicomponent approach to identify the unique botanical composition, physicochemical properties and antibacterial activity of selected honeys produced from a unique floral suite along the West Coast of South Africa. The overarching goal of this study was to identify distinct characteristics of honeys produced from indigenous CFR vegetation (hereafter also referred to as fynbos), which could appeal to elite local honey consumers and help to facilitate international trade. This could, in turn, help financially sustain the struggling South African beekeeping industry.

Study design

Industry partner

This study was designed in conjunction with Hurters Honey (Langebaan, South Africa), the industry partner on this project. The data obtained from investigating the botanical origin, the physicochemical properties and the antibacterial activity of selected West Coast honey will feed back into marketing Hurters Honey to local as well as international honey consumers.

Study area

A 40 km stretch along the West Coast of South Africa, consisting of a mosaic of two CFR vegetation types, namely the Hopefield sand fynbos and Saldanha flats strandveld, were selected to conduct this study (SANBI 2012). These are unique vegetation types, characterised by the presence of many endemic fynbos plant species (Rebelo et al. 2006; Helme 2007), and therefore the suite of flowering plants present in this area could potentially deliver distinctive honeys. Throughout this document, this study area is broadly referred to as the West Coast. Besides the unique vegetation this area offers, it was also chosen because most of the beekeepers in this region deliver their honey harvests to Hurters Honey. The specific apiary sites within the area selected for this study have also historically produced commercially viable honey flows that could potentially produce monofloral honeys during peak bloom for particular plant species. Thus, it made sense to focus this study on this area of the West Coast, as the unique vegetation that is available for honey production will be exclusive to Hurters Honey products.

A total of 36 *A.m. capensis* colonies in Langstroth beehives were loaned to the project by beekeepers who have permanent apiary sites along the West Coast. Six apiary sites with six colonies each were distributed along the 40 km stretch, with all apiaries at least 10 km apart: Boplaas and Middelkraal in the north, Kersefontein 1 and 2 in the centre, and Thali Thali and Hopefield in the south (Figure 1). For the purposes of this study, all the hives were refurbished and fed with sugar preceding the first honey harvest of each year to ensure similar colony strength and hive conditions between the apiary sites.

Honey harvests

Honey harvests took place over a three-year period (2015 to 2017) from the beginning of September to the middle of December each year. No major alien or agricultural crops flowered during this four-month period. Each of the 36 hives across the six sites were assigned specific honey supers with individually marked frames to keep track of each hive's honey production and honey extractions over the sampling period. Each frame in each super from every hive was monitored on a two-weekly basis. A grid overlay system was used to determine whether a frame of honey was ready for extraction. If 70% of the honey on the frame was ripe and capped it was removed for extraction and replaced with an empty frame assigned to that hive.

All the harvested frames from an apiary site from a two-week period were extracted together, creating a composite sample for the six hives present at that site. This combined sample 1) reflects the overall resource use across the landscape, eliminating individual colony foraging preference and 2) is more similar to commercial beekeeping practices, with beekeepers harvesting and bulking their honeys from different hives. A second sampling regime was applied during periods of high honey flow. When frames from a specific apiary site were empty at the beginning of a two-week sampling period and were filled and capped within that period, these frames were extracted separately for that site. This high honey flow period was then correlated with the specific plants flowering during the same period and evaluated as a potential monofloral honey. Honeys were harvested as raw, i.e. never heated and only strained using gravity. The frames from each apiary site were always handled separately during the extraction process. This method ensured small batch honey harvests to capture the presence of the specific unique plant species flowering in two-week windows, potentially increasing the amount of monofloral honey harvests.

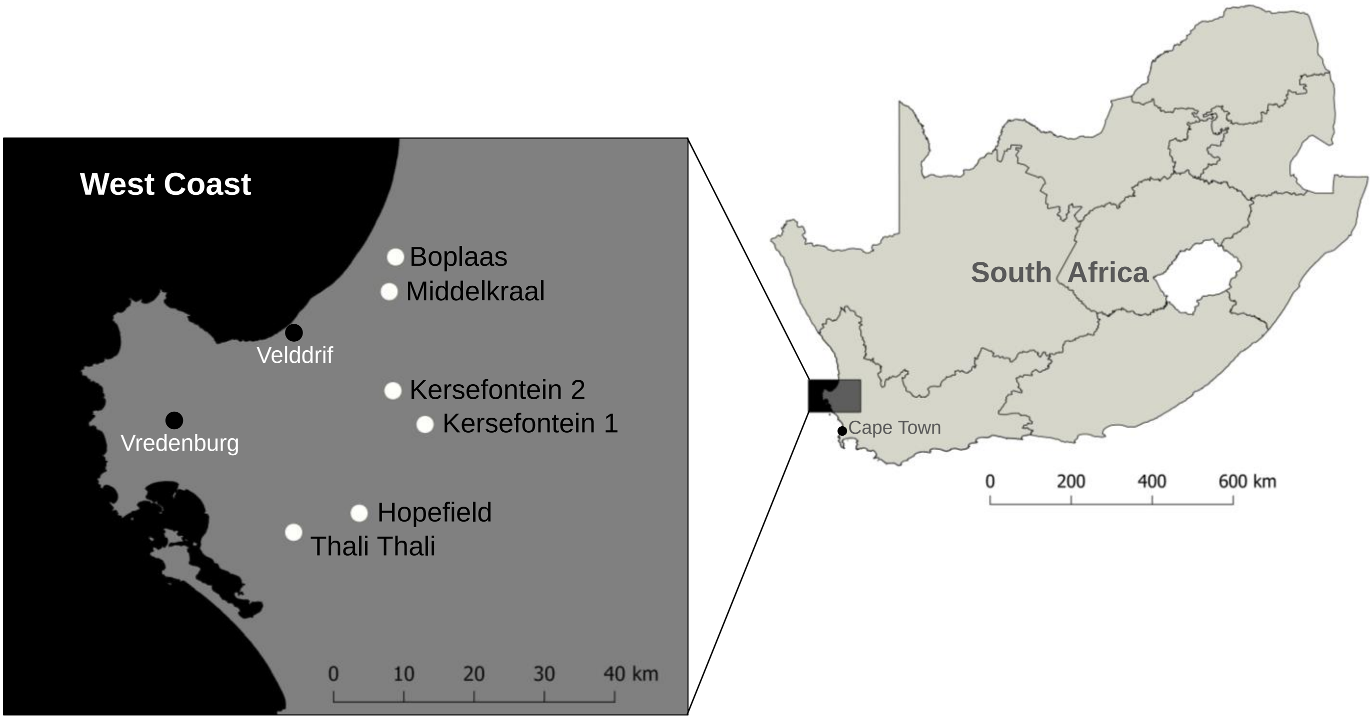

Figure 1: A map of South Africa indicating the West Coast region and a close-up of the area indicating the six apiary sites included in this study: Boplaas and Middelkraal in the north, Kersefontein 1 and 2 in the centre, and Thali Thali and Hopefield in the south. The town names of Cape Town, Vredenburg and Velddrif are indicated as reference points. Maps were drawn in QGIS 2.18.9 (QGIS Geographic Information System, Open Source Geospatial Foundation Project).

Aims and Objectives

This dissertation follows the format of a series of scientific papers. The dissertation is divided into five chapters, including the current General Introduction chapter. The specific aims and objectives of the remaining four chapters are:

Chapter 2. The botanical origin of selected West Coast honeys: Novel findings and challenges using melissopalynology.

Aim: To determine the botanical origin and composition of selected West Coast honeys

Objective 1: Generate the first pollen library for the West Coast region of South Africa

Objective 2: Determine the botanical composition of the honey using melissopalynology

Objective 3: Identify the most important plant species represented in the honey samples.

Chapter 3. The physicochemical properties of selected West Coast honeys and how these properties change with honey age.

Aim: To evaluate the physicochemical properties of selected West Coast honeys

Objective 1: Determine whether the honey meets international legal requirements

Objective 2: Analyse the relationship between the botanical composition and physicochemical properties of the honeys

Objective 3: Assess how the honey properties change over time with honey age.

Chapter 4. The antibacterial activity of selected West Coast honeys against *Staphylococcus aureus* bacteria.

Aim: To assess the antibacterial properties of selected West Coast honeys

Objective 1: Determine the phenol equivalent antibacterial activity of the honey samples

Objective 2: Analyse the relationship between the botanical composition and antibacterial activity of the honeys

Objective 3: Assess how the antibacterial activity of the honey changes with honey age.

Chapter 5. Honey production along the West Coast: perspectives and suggestions for the South African beekeeping industry.

Aim: To assess the historical trends of honey production along the West Coast in relation to annual rainfall, and to discuss beekeeping strategies for the region going forward with the potential of increasing droughts. The discussion also highlights outcomes of previous chapters and the application thereof for West Coast beekeepers and the South African beekeeping industry.

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Chapter 2. The botanical origin of selected West Coast honeys: Novel findings and challenges using melissopalynology.

Introduction

Honey is a sweet natural product made by honey bees from a variety of sugary plant fluids. The raw material used to produce natural honey is either collected as nectar secreted from floral or extrafloral nectaries, or as honeydew secreted by sapsucking insects (Maurizio 1975a; Doner 1977). Honey is highly variable and differs significantly between geographic regions, years and seasons. This is largely due to different vegetation types and fluctuating floral availability (White et al. 1962; El-Sohaimy et al. 2015). In the case of floral origin or "blossom" honeys, foraging worker honey bees collect the nectar secreted by flowering plants and ripen this in the hive through the addition of enzymes and a process of evaporation. Even though compounds like enzymes are added by the bees themselves during the honey making process, the specific chemical composition and characteristics of each honey with regards to organoleptic properties, are primarily influenced by the distinct nectar composition of the plants that were visited by the bees (White et al. 1962; Crane 1975a; Persano Oddo and Piro 2004). Therefore, honeys produced in different geographic areas and in different years or seasons often have different botanical compositions and unique properties associated with specific nectar collected by the honey bees.

Characterising the botanical composition of honey is very important for marketing purposes. In recent years the global trend in the food market has moved towards origin-based marketing of products that appeal to certain segments of consumers whose decision to buy is often influenced by preferences for high quality foods of specific varieties (Engelbrecht et al. 2014) or certain geographic areas of production (McCluskey and Loureiro 2003). These groups of consumers are likewise increasingly sourcing sustainably and locally produced foods, showing a particular interest in the quality, authenticity and food safety of the products they buy (Jekanowski et al. 2000; Loureiro and McCluskey 2000; Michaelidou and Hassan 2008; Adams and Salois 2010). In line with the global trend in origin-based marketing and traceability of food, certain elite consumers of honey are willing to pay more for locally produced honeys and honeys with a specific botanical origin (Crane 1975b; Unnevehr and Gouzou 1998; Wu et al. 2015; Cosmina et al. 2016).

Many different methods are used to compare the chemical signatures of honeys with specific botanical compositions to verify the botanical origin of honey. These include techniques such as near-infrared spectroscopy (Escuredo et al. 2015), mid-infrared spectroscopy (Ruoff et al. 2006), nuclear magnetic resonance spectroscopy (Schievano et al. 2012), Raman spectroscopy (Oroian and Ropciuc

2017), gas chromatography-mass spectrometry (GC-MS) (Manyi-Loh et al. 2011) and electronic tongue techniques (Elamine et al. 2019). However, these methods are only useful to distinguish between honey varieties with unique chemical composition fingerprints that have already been characterised. They require a considerable amount of preliminary work on multiple samples from the same known botanical origin to build up the databases for identifying chemical signatures that are unique to specific honey varieties (Ruoff et al. 2006) and are therefore impractical for identifying novel honey samples. Before the chemical signatures of an undescribed honey variety can be useful for verification of similar samples, the botanical composition of the variety must first be determined through the identification and quantification of the different plant species' pollen grains that are trapped in the honey.

The idea behind the botanical identification of honey through pollen analysis is that the honey stored in a colony contains pollen grains from the flowers it originated from, proportional to the number of visitations that plant species received from the foraging bees (Maurizio 1975b). There are three ways in which pollen from different floral sources are trapped in a honey sample: 1) The primary and most direct route of transfer is via the nectar in the honey stomach of the foraging honey bee, i.e. when the honey bee visits a floral nectar source and pollen from the anthers fall into the nectar before it is sucked up my the bee and transported back to the hive. 2) A smaller amount of secondary pollen addition to honey can take place when pollen stuck to the body of honey bees is transferred to the ripening honey in open cells in the hive or when pollen grains floating in the air are transported into the ripening honey. 3) Finally, tertiary pollen addition can be caused by the beekeeper during honey extraction and processing when honey frames containing many pollen cells are extracted or when honeys from different origins are extracted in the same extractor (Maurizio 1975b; Sawyer 1988; Bryant and Jones 2001; Von der Ohe et al. 2004). The latter two ways of pollen enrichment could potentially introduce pollen grains to the honey that do not correspond to the nectar sources utilized to produce the honey.

One method of identifying the specific botanical origin of pollen grains trapped in honeys is DNA barcoding. The DNA of different plant species is identified, and the relative contribution of each plant can be measured with quantitative PCR techniques (Schnell et al. 2010; Bruni et al. 2015; Hawkins et al. 2015). Even though this method is very fast and accurate, it is expensive and relies on the building of a complete DNA reference database of all the potential plant species contributing to a honey sample (Cowan and Fay 2012; Stein et al. 2014). Another method for determining the botanical origin of honey is melissopalynology, which is an established technique employed since the early 1930's (Maurizio 1975b). With this low-cost technique, the pollen components of the honey are investigated using light microscopy. Pollen from different plant species can be characterised by their shape, size, features of the exine (the hard outer coating surrounding the pollen grain) and apertures. Apertures are the openings in the exine through which the pollen tubes will emerge and grow during the process of angiosperm fertilization (Punt et al. 2007). The number, position and form of these apertures are important in distinguishing pollen grains from different plant taxa. The thickness and surface of the exine is also characterised by different structures such as knobs, spines, rods, etc. (Erdman 1952). All of these characteristics are used to identify the different pollen grains trapped in honey (Sawyer 1981; Sawyer 1988).

The ability to identify pollen contained in honey can be used to verify the geographic and botanical origin of honey and to classify the botanical composition more accurately as being either monofloral or multifloral (Von der Ohe et al. 2004). Monofloral honeys contain at least 45% of pollen grains from a single plant species. However, it is challenging to quantify the contribution of different plant species to the botanical origin of honeys, since the relative pollen counts of plant species might be an under- or over-representation of the actual nectar contribution to the honey (Bryant and Jones 2001; Rodopoulou et al. 2018). Flower morphology will influence the number of pollen grains present in a certain volume of honey through to the number of anthers present, the amount of pollen produced and the openness of the nectaries that catch the falling pollen (Maurizio 1975b). Pollen estimates therefore do not always reflect the correct proportion of nectar originating from a specific plant. Furthermore, during flight the foraging honey bee could also lose pollen grains through the proventriculus filtering out pollen grains from the honey stomach contents (Dade 2009). This means that the further the floral resources are located from the hive or the longer a forager takes to return to the colony, the greater the chance of pollen reduction in the nectar load. This is a limitation to keep in mind when interpreting the results of pollen quantification through DNA analyses or melissopalynology.

The standardised melissopalynology method originally proposed by Louveaux et al. (1978) and later amended by Von der Ohe et al. (2004) has been widely used, although sometimes modified, to test the botanical origin and composition of honeys from countries all over the world, including Argentina (Naab et al. 2008), Canada (Crompton and Wojtas 1993), Ethiopia (Belay et al. 2015), Finland (Salonen et al. 2009), India (Ponnuchamy et al. 2014), New Zealand (Moar et al. 1985), Nigeria (Ebenezer and Olugbenga 2010) and the United States of America (Jones and Bryant 2014). South Africa is well-known for its high biodiversity and the Western Cape Province is especially famous for its large variety of plant species. The Cape Floristic Region (CFR) biodiversity hot spot, located in the Western Cape Province, is the smallest of the six recognized floral kingdoms of the world (Good 1947), but it contains more than 9000 plant species of which 70-80% are endemic (Manning and Goldblatt 2012). With this in mind, honeys produced in the CFR region of South Africa should also have their own distinctive organoleptic properties and characteristics that reflect the area's unique floral bouquet. Despite this potential for origin-based marketing of unique honeys, currently the South African honey market is not orientated towards the marketing of niche products and the botanical sources contributing to honey harvests are not characterised.

Most honeys from the CFR region are currently harvested in bulk across many apiary sites with different plant species compositions and sold as a composite regional product or as fynbos honey. This is a missed opportunity to market potential monoflorals from plant species that are unique to this region and could potentially be marketed at a premium price to elite local and international honey consumers. Identifying monoflorals in the CFR region with such a large number of plant species is, however, rather challenging. Although the DNA of many groups of fynbos plants have been sequenced to date (e.g. Verboom et al. 2009), the relevant loci from all the plant species occurring in any given area of honey production will have to be sequenced to obtain a complete DNA barcoding reference library for the botanical classification of honey. This is not always feasible, as DNA analysis is a very expensive method. The classical method of analysing the botanical composition of honey, melissopalynology, is thus often more appropriate.

To date there is only one publication that used melissopalynology to investigate the botanical origin of selected honeys from South Africa (Johannsmeier 2001a). His broad scale approach shed light on some of the important plant families for honey production in the country. Yet a fine scale study of the botanical origin and composition of South African honeys at a local scale has not been undertaken. This study aims to establish the first local pollen library for the West Coast region of South Africa and to use melissopalynology to determine the floral composition of honeys produced in this area. The most important plant species represented in the honey samples will be identified and honeys will be classified as being multi- or monofloral. Any monofloral honeys produced from unique, endemic plant species could present an opportunity for beekeepers to optimise their production at sites where these plants occur in abundance and to increase their income through origin-based marketing to local and international consumers.

Methods

Study area and honey samples

Fieldwork took place over a three-year period (2015 to 2017) from the beginning of September to the middle of December each year. During this time a total of 36 honey bee colonies in Langstroth beehives were managed across a 40 km stretch along the West Coast of South Africa. The area consisted of six apiary sites with six colonies each: Boplaas and Middelkraal in the north, Kersefontein 1 and 2 in the centre, and Thali Thali and Hopefield in the south (Chapter 1, Figure 1). These specific apiary sites were chosen since they consist predominantly of indigenous fynbos vegetation and have historically produced commercially viable honey flows.

All the frames in each super from every hive at all six apiary sites were monitored on a two-weekly basis and frames were harvested when they were at least 70% capped. This method ensured small batch honey harvests to temporally align and capture the presence of the specific plant species flowering in that two-week window, increasing the likelihood of finding monofloral honeys in a diverse landscape. Frames from each apiary site were handled separately during the extraction process, during which honeys were never heated and only strained using gravity. Sixty-six honey samples were collected over 2015 (n = 3), 2016 (n = 33) and 2017 (n = 30). The samples came from the different apiary sites as follows: Boplaas (n = 19), Middelkraal (n = 8), Kersefontein 1 (n = 10), Kersefontein 2 (n = 10), Thali Thali (n = 12) and Hopefield (n = 7). Honey sample sizes were low due to an ongoing drought, particularly in 2015, with only three honey samples collected from two of the apiary sites.

Plant collection and identification

From the last week of August until the middle of December each year, all flowering plants species within a 3 km radius around the six apiary sites were sampled and pressed. Plant species were identified by the Compton Herbarium at Kirstenbosch Botanical Gardens and the Bolus Herbarium at the University of Cape Town (Cape Town, South Africa), as well as by botanists at Stellenbosch University (Stellenbosch, South Africa). On a two-weekly basis, honey bees were actively searched for at each apiary site in a 1.5 km radius around the hives to determine the flowering plants they visited most (hereafter called bee-plants) and what resource they were foraging for – nectar, pollen or both. This distance of 1.5 km is based on the mean foraging distance of bees during times of resource abundance, which is known to range between 0.6 km and 1.5 km (Beekman and Ratnieks 2000; Beekman et al. 2004; Danner et al. 2016). Honey bees found foraging on plants outside of this active search area were also documented.

West Coast pollen library

Stamens or whole flowers from each plant species identified in the 3 km radius around the six apiary sites were collected in Eppendorf tubes and stored at -18°C for use in the palynology section of this study. Stamens were dissected to collect pollen, which was then fixed onto microscope slides to make pollen reference slides of each species. The pollen was mounted onto the slides using a glycerin jelly and stained using fuchsin staining (Delaplane et al. 2013). A light microscope with camera (DMS500, Leica Microsystems, Germany) was also used to photograph the pollen slides and generate an electronic reference collection. The size of pollen grains from different plant species was measured using the Leica Application Suite software (LAS, Leica Microsystems, Germany).

Subset pollen libraries

Using the complete West Cost pollen library to identify pollen grains trapped in the different honey samples was challenging, and consequently the library was further subdivided into smaller libraries based on the flowering time and flowering location of the different plant species. This spatially and temporally aligned specific honey samples with the potential pollen grains that could be present in that sample, based on the time and location of the honey harvest. This system optimised the pollen identification process.

Melissopalynology

The pollen trapped in each honey sample were isolated and examined according to the method of qualitative melissopalynological analysis proposed by Van der Ohe et al. (2004): 10 g of honey per sample was weighed using an analytical balance (Radwag XA 110/Y, Radwag Balances and Scales, Poland), dissolved in 20 ml distilled water and centrifuged for 10 minutes at 1000 g (Heraeus Biofuge Stratos Centrifuge, Thermo Fisher Scientific, USA). Thereafter the supernatant liquid was discarded, and the sediment layer was again dissolved in 20 ml distilled water and centrifuged for 5 minutes at 1000 g. The supernatant liquid was again discarded, and the leftover sediment was used to prepare permanent pollen microscope slides using the glycerin jelly method with fuchsin staining (Van der Ohe et al. 2004; Delaplane et al. 2013).

From these honey pollen slides the relative frequencies of the pollen of different plant species were quantified by counting and identifying approximately 1000 evenly distributed pollen grains per slide (mean: 1186.23 ± 386.53 SD), using the subset pollen libraries compiled from the plants species in the area. The relative frequencies of each pollen type were calculated as the percentage of the total number of pollen grains counted. The relative pollen presence in honey can be described either as 1) Predominant: a species that constitutes 45% of pollen grains; 2) Secondary dominant: a species that constitutes 16-44% of pollen grains; 3) Important minor pollen: a species that constitutes 3-15% of pollen grains; or 4) Minor pollen: a species that constitutes less than 3% (Sawyer 1988). The honey samples were classified as being either monofloral or multifloral, where monofloral samples were defined as having at least 45% of its pollen from a single floral origin. For the sake of brevity, only the plant species that made up more than 3% of each sample are reported here.

Assessment of bee-plant availability

During the last year of fieldwork, ten GPS coordinates within the 1.5 km radius around each apiary site were randomly selected from a larger set of random coordinates plotted in Garmen BaseCamp version 4.6.2 (Garmin Ltd., USA) to assess the availability of bee-plants at the different apiary sites. At each GPS point, percentage coverage of 23 selected bee-plants was assessed within a 10 x 10 m quadrat. The area in m^2 occupied by each plant species within each 100 m^2 quadrant at each apiary site was measured using a measuring tape and converted to percentage coverage. The 23 plant species were chosen based on their attractiveness to honey bees, as assessed through observations of honey bee forging across all sites during the first two years of fieldwork (Table S1).

Statistical analyses

The botanical compositions of the 66 honey samples harvested over the three years at the different sites were compared using multivariate analyses in PRIMER version 6 (Plymouth Routines in Multivariate Ecological Research, Plymouth Marine Laboratory, UK). Only pollen types that made up 3% or more of any specific honey sample were included in the dataset. These data were fourth root transformed and a resemblance matrix was constructed using Bray-Curtis similarity. The botanical composition of the different honey samples was analysed with a principal coordinates analyses (PCoA), with vectors of botanical species overlaid on each PCoA if that pollen type had a Pearson's correlation of $r \geq 0.6$ to PC1 or PC2. A Permutational Multivariate Analysis of Variance (PERMANOVA) was also run on the Bray-Curtis resemblance matrix, with site included as a fixed factor (Boplaas = B, Middelkraal = M, Kersefontein 1 = K1, Kersefontein 2 = K2, Thali Thali = TT, Hopefield = H) and year (2015, 2016, 2017) as a random factor nested within site. The model was defined as such, because comparing years only makes sense within a specific apiary site (see e.g. Ponnuchamy et al. (2014)). Pair-wise t-tests were done to determine specific differences, if a factor was found to be significant in the model. A similarity percentages (SIMPER) analysis was performed on the fourth root transformed data to identify the most important pollen types contributing to the honey samples from each site, as well as any differences found between sites.

A pollen diagram, summarising the contributions of each pollen type to the different honey samples, was constructed using the rioja package in RStudio version 1.2.1335 (RStudio Inc., USA). For the purpose of clarity, only the pollen types that made up 10% or more of any specific honey sample was included.

The bee-plant availability at the different sites was investigated by comparing percentage plant coverage between the six apiary sites. Only plant species that made up at least 3% of any 100 m^2 quadrat sampled at any of the sites were included in the analyses. These data were analysed in PRIMER version 6 (Plymouth Routines in Multivariate Ecological Research, Plymouth Marine Laboratory, UK), square root transformed, and a resemblance matrix was constructed using Bray-Curtis similarity. A PERMANOVA was run on the Bray-Curtis resemblance matrix, with site included as a fixed factor. All significant differences are based on $p < 0.05$.

Results

Pollen library and palynology

Two hundred and thirty-three plant species from 51 families were identified within the study area and included in the first pollen library for the West Coast of South Africa. Using this library, 108 plant species and morphotypes (hereafter referred to collectively as pollen types) from 38 plant families were identified within the honey samples across all sites, years and harvests. The families and pollen types that made up more than 1% of the total counted pollen trapped in the honey samples are summarised in Figure 1. The five most abundant families present in the honey samples were: Fabaceae (26.86%), Zygophyllaceae (15.67%), Aizoaceae (14.71%), Apiaceae (8.94%) and Asphodelaceae (4.15%). Pollen types, where the family could not be identified with certainty, made up only 1.37% of the total grains counted in all honey samples. The five most abundant pollen types identified were: *Zygophyllum morgsana* L. (14.76%), *Aspalathus spinescens* Thunb. (form A) (14.32%), *Conicosia / Carpanthia* type (13.82%), *Capnophyllum africanum* (L.) Gaertn. (8.94%) and a *Trachiandra* sp. (4.19%). These five types made up 56.75% of the pollen identified in all the honey samples. The pollen types that were labelled as unknown, not resembling any pollen families or species in the library, made up 0.22% of the total number of pollens counted.

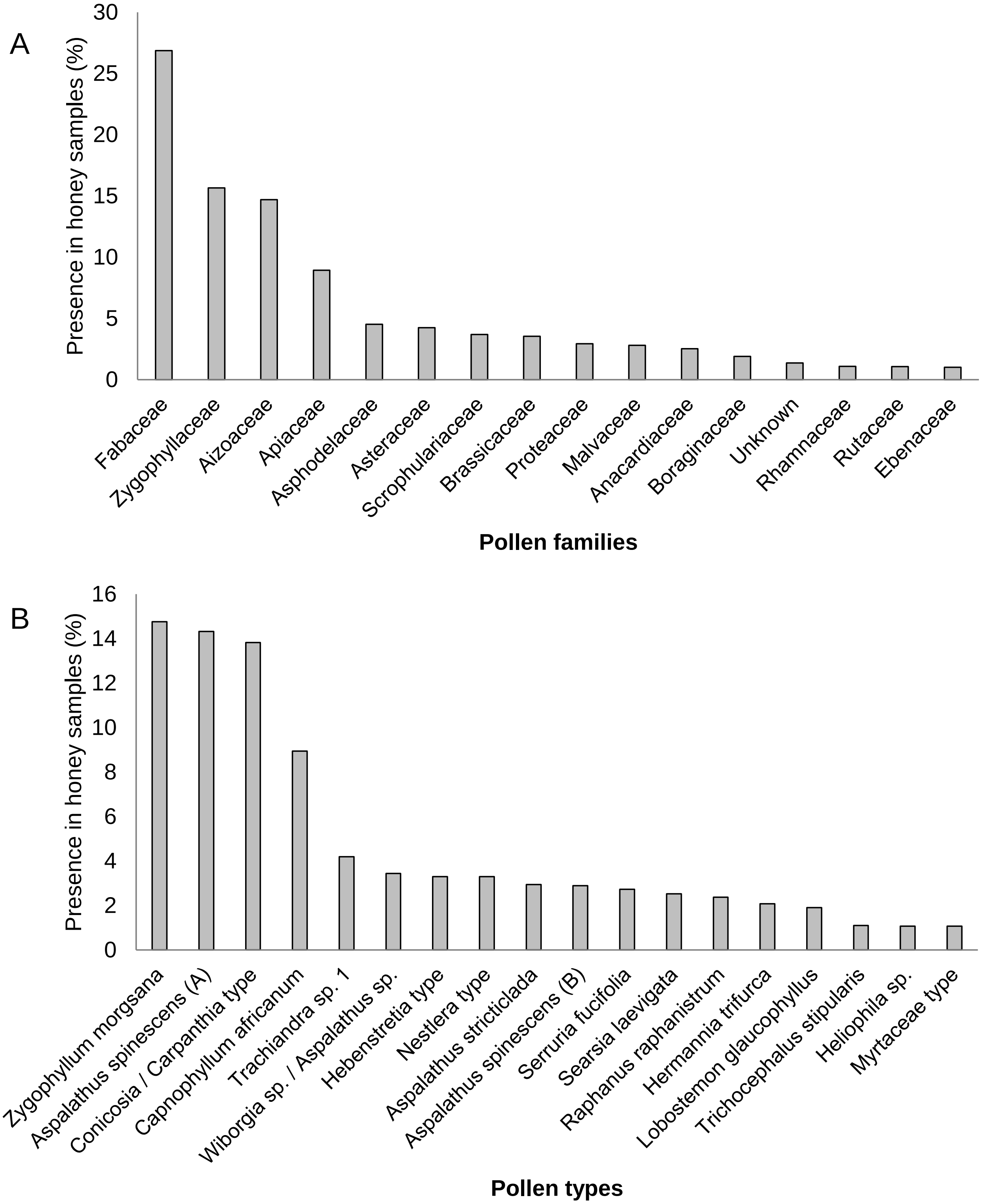

Figure 1: Summary of the most prevalent families (A) and pollen types (B) found in honey samples from the West Coast. Taxa were included if they contributed more than 1% to the total pollen count across all samples.

Honey composition between sites

Figure 2 is a pollen diagram illustrating the botanical composition of honey samples. The composition of honey samples differed significantly between sites (PseudoF = 2.88, df = 5, p = 0.002). Yet the differences in botanical compositions of honey samples between sites were only significant for Boplaas vs. Kersefontein 2 (t = 2.50, p = 0.049) and Boplaas vs. Thali Thali (t = 2.00, p = 0.04), with very different pollen types contributing to the honeys from these sites (Table 1).

Table 1: Average percentage dissimilarity (%) of the most important pollen types contributing to the dissimilarity between sites that had honeys with different botanical origins. Only those pollen types that reached a cumulative contribution of 50% dissimilarity (SIMPER analysis) were included. Boplaas = B, Kersefontein 2 = K2, Thali Thali = TT.

Site	Dissimilarity (%)	Pollen types	Cumulative %
B vs. K2	80.87	*Aspalathus spinescens* (form A)	12.45
		Conicosia / Carpanthia type	24.65
		Capnophyllum africanum	36.14
		Raphanus raphanistrum	46.73
		Wiborgia / Aspalathus sp.	56.05
B vs. TT	86.22	*Aspalathus spinescens* (form A)	12.57
		Conicosia / Carpanthia type	24.60
		Aspalathus stricticlada	34.42
		Zygophyllum morgsana	42.38
		Hebenstretia type	49.62
		Nestlera type	55.81

The differences in composition of honeys across the different sites can clearly be ascribed to the separation of honeys harvested from Boplaas to those from Kersefontein 2 and Thali Thali (Figure 3). Honeys from Boplaas are dominated by pollen of *A. spinescens* (form A) and *Conicosia / Carpanthia* type (Figure 3, Table 2). This is further supported by the SIMPER analysis where *Conicosia / Carpanthia* type was identified as the most important plant type defining honey samples harvested at Boplaas and Kersefontein 1, contributing 43.03% and 40.09% to the similarity between samples at these sites, respectively (Table 2). For Middelkraal, *A. spinescens* (form A) contributed 34.53% to the similarity of honey samples and for Kersefontein 2, *C. africanum* was the most important defining plant type contributing 36.77% (Figure 3, Table 2). *Aspalathus stricticlada* (R.Dahlgren) R.Dahlgren (27.31%) and *A. spinescens* (form B) (32.94%) were the pollen types contributing the most to the similarity of honey samples at Thali Thali and Hopefield, respectively (Table 2).

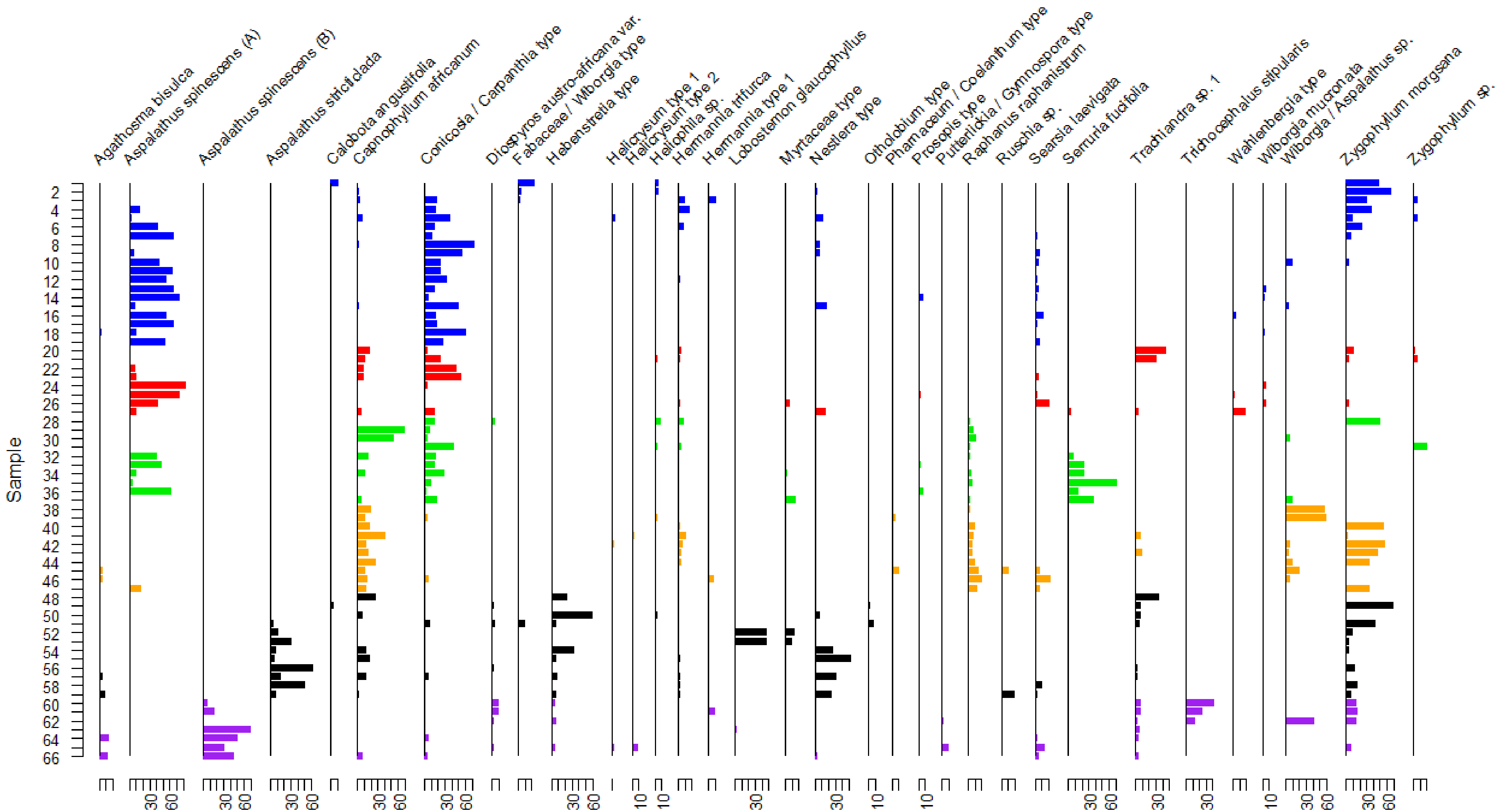

Figure 2: Pollen diagram showing the percentage of each plant taxon (x-axis) present in the 66 honey samples from the West Coast. Only plant taxa that contributed 10% or more to any honey sample were included. Samples 1 to 19 = Boplaas (blue bars), Samples 20 to 27 = Middelkraal (red bars), Samples 28 to 37 = Kersefontein 1 (green bars), Samples 38 to 47 = Kersefontein 2 (orange bars), Samples 48 to 59 = Thali Thali (black bars) and Samples 60 to 66 = Hopefield (purple bars).

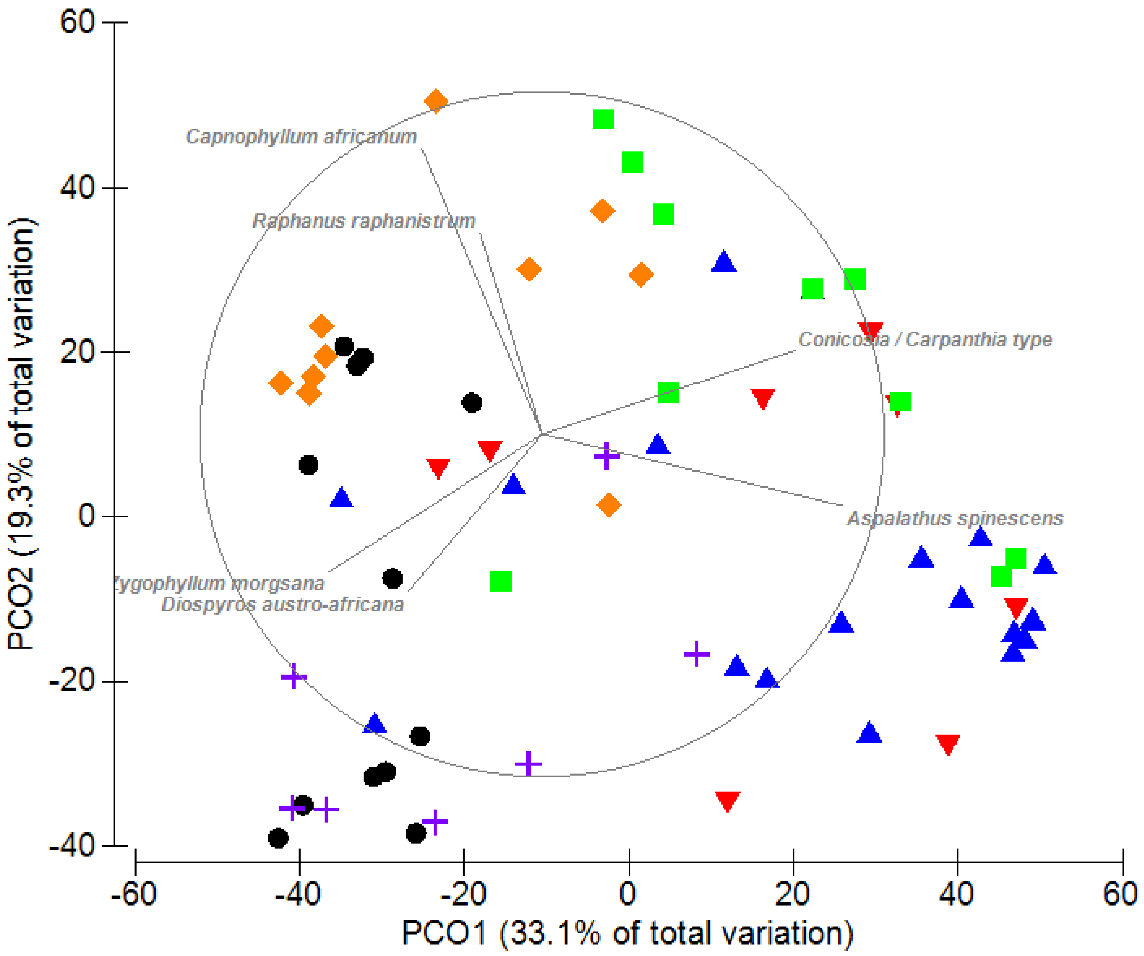

Figure 3: Principal Coordinates Analysis showing the total variance (52.4%) of the botanical composition of honeys harvested along the West Coast. Honey compositions correlate with vector overlay based on Pearson's correlation of $r \geq 0.6$. The length of the vector indicates the importance of the plant species to the honey composition. Sites are indicated as Boplaas = B (blue triangles), Middelkraal = M (red inverted triangles), Kersefontein 1 = K1 (green squares), Kersefontein 2 = K2 (orange diamonds), Thali Thali = TT (black circles) and Hopefield = H (purple crosses).

Honey composition between years

The botanical composition of honeys harvested within the sites differed across the harvest years (PseudoF = 4.19, df = 8, p = 0.001) in 2016 and 2017, but not in 2015. During 2015 honey was only harvested from two sites due to the drought. Differences in honey composition was found between 2016 and 2017 at Boplaas (t = 2.21, p = 0.004), Kersefontein 2 (t = 1.90, p = 0.028), Thali Thali (t = 3.00, p = 0.003) and Hopefield (t = 2.85, p = 0.04).

Table 2: Similarity percentages (SIMPER) analysis showing the species that contributed most to the similarity of samples within each site. Contributing species were included until a cumulative site contribution to similarity of 90% had been reached. Boplaas = B, Middelkraal = M, Kersefontein 1 = K1, Kersefontein 2 = K2, Thali Thali = TT, Hopefield = H.

Site	Pollen type	Family	Contribution (%)	Cumulative %
B	*Conicosia / Carpanthia* type	Aizoaceae	43.03	43.03
	Aspalathus spinescens (form A)	Fabaceae	35.16	78.19
	Searsia laevigata	Anacardiaceae	8.13	86.31
	Zygophyllum morgsana	Zygophyllaceae	6.84	93.15
M	*Aspalathus spinescens* (form A)	Fabaceae	34.53	34.53
	Conicosia / Carpanthia type	Aizoaceae	27.55	62.08
	Capnophyllum africanum	Apiaceae	16.78	78.86
	Searsia laevigata	Anacardiaceae	5.08	83.94
	Trachiandra sp. 1	Asphodelaceae	4.53	88.47
	Zygophyllum morgsana	Zygophyllaceae	3.83	92.30
K1	*Conicosia / Carpanthia* type	Aizoaceae	40.09	40.09
	Raphanus raphanistrum	Brassicaceae	20.04	60.14
	Serruria fucifolia	Proteaceae	15.68	75.81
	Aspalathus spinescens (form A)	Fabaceae	10.62	86.43
	Capnophyllum africanum	Apiaceae	9.99	96.42
K2	*Capnophyllum africanum*	Apiaceae	36.77	36.77
	Raphanus raphanistrum	Brassicaceae	24.38	61.14
	Wiborgia / Aspalathus sp.	Fabaceae	15.11	76.25
	Zygophyllum morgsana	Zygophyllaceae	13.48	89.73
	Hermannia trifurca	Malvaceae	6.17	95.90
TT	*Aspalathus stricticlada*	Fabaceae	27.31	27.31
	Zygophyllum morgsana	Zygophyllaceae	21.08	48.39
	Hebenstretia type	Scrophulariaceae	14.59	62.98
	Capnophyllum africanum	Apiaceae	10.45	73.43
	Trachiandra sp. 1	Asphodelaceae	9.84	83.26
	Nestlera type	Asteraceae	7.58	90.85
H	*Aspalathus spinescens* (form B)	Fabaceae	32.94	32.94
	Trachiandra sp. 1	Asphodelaceae	24.10	57.04
	Zygophyllum morgsana	Zygophyllaceae	10.41	67.44
	Searsia laevigata	Anacardiaceae	9.00	76.44
	Diospyros austro-africana var. *rugosa*	Ebenaceae	8.23	84.67
	Trichocephalus stipularis	Rhamnaceae	6.11	90.79

Monofloral honey varieties

Of the 66 honey samples, 39 were identified as being monofloral based on their pollen counts across all years and sites. These samples were dominated by pollen of 11 different plant species: *A. spinescens* (form A) (n = 13), *A. spinescens* (form B) (n = 3), *A. stricticlada* (n = 2), *C. africanum* (n = 2), *Conicosia / Carpanthia* type (n = 6), *Hebenstretia* type (n = 1), *Lobostemon glaucophyllus* (Jacq.) H.Buek (n = 2), *Nestlera* type (n = 1), *Serruria fucifolia* Salisb. ex Knight (n = 1), a *Trachiandra* sp. (n = 1) and *Z. morgsana* (n = 7). The seven monofloral varieties produced in more than one year or at multiple sites (i.e. n ≥ 2) are summarised in Table 3 and the pollen grains from these plant species are shown in Figure 4.

Table 3: The monofloral honey varieties produced in more than one year or at multiple sites (i.e. n ≥ 2) along the West Coast of South Africa.

Plant species	Family	Samples	Years	Sites
Aspalathus spinescens (form A)	Fabaceae	13	2015	Boplaas
			2016	Kersefontein 1
			2017	Boplaas
				Middelkraal
				Kersefontein 1
Aspalathus spinescens (form B)	Fabaceae	3	2016	Hopefield
Aspalathus stricticlada	Fabaceae	2	2017	Thali Thali
Capnophyllum africanum	Apiaceae	2	2016	Kersefontein 1
Conicosia / Carpanthia type	Aizoaceae	6	2016	Boplaas
				Middelkraal
Lobostemon glaucophyllus	Boraginaceae	2	2017	Thali Thali
Zygophyllum morgsana	Zygophyllaceae	7	2016	Boplaas
			2017	Boplaas
				Middelkraal
				Kersefontein 1
				Kersefontein 2
				Thali Thali

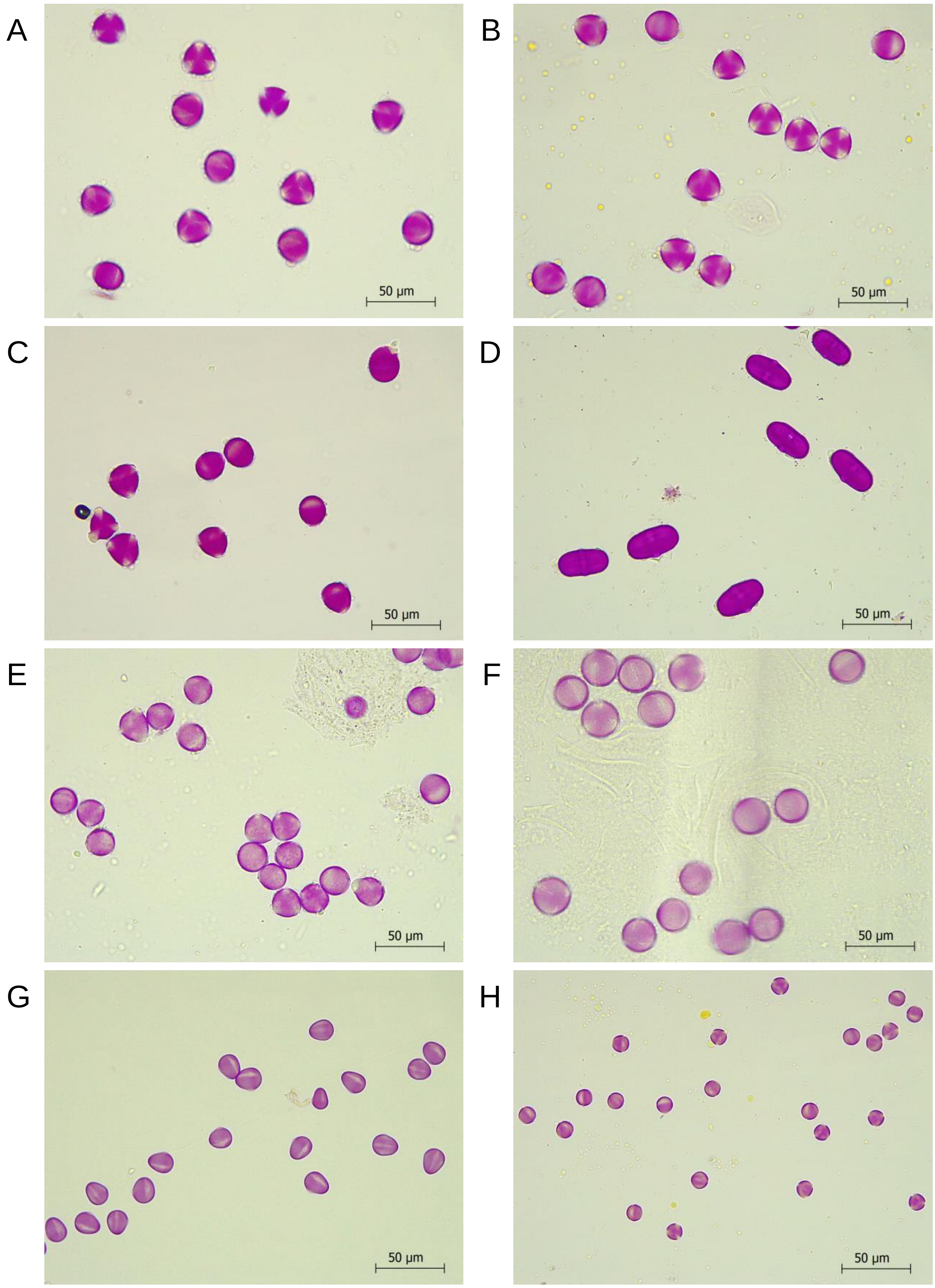

Figure 4: Pollen grains of the plant species that make up the seven monofloral honey varieties harvested along the West Coast of South Africa. A) *Aspalathus spinescens* (form A), B) *Aspalathus spinescens* (form B), C) *Aspalathus stricticlada*, D) *Capnophyllum africanum*, E) *Conicosia* sp. and F) *Carpanthia* sp. (*Conicosia* / *Carpanthia* type), G) *Lobostemon glaucophyllus*, H) *Zygophyllum morgsana*.

Assessment of bee-plant availability

The plant species percentage coverage assessed at the six apiary sites differed significantly (Pseudo-F = 4.613, df = 5, p = 0.001). Subsequent pair-wise t-tests showed that there was a significant difference in plant composition between most sites, except for Boplaas and Middelkraal (t = 1.06, p = 0.361) and Kersefontein 2 and Middelkraal (t = 1.40, p = 0.088) (Table 4).

Table 4: Results from a pair-wise t-test comparison of overall plant species composition at the different apiary sites. Boplaas = B, Middelkraal = M, Kersefontein 1 = K1, Kersefontein 2 = K2, Thali Thali = TT, Hopefield = H. Bold p-values indicate significance ($p < 0.05$).

Site	B	M	K1	K2	TT
M	0.361				
K1	**0.001**	**0.001**			
K2	**0.006**	0.088	**0.001**		
TT	**0.002**	**0.001**	**0.001**	**0.002**	
H	**0.002**	**0.002**	**0.001**	**0.002**	**0.001**

Discussion

This is the first comprehensive pollen library compiled for the West Coast of South Africa. By adopting a melissopalynological approach, the major plant species that honey bees use to produce honey in this area were identified. Honey bees produced monofloral honeys from seven plant species that differed across time and space. In general, monofloral honey varieties are more sought after and fetch higher prices on the international honey market compared to uncharacterised multifloral honeys (Unnevehr and Gouzou 1998). Thus, adopting a relatively easy and inexpensive method like melissopalynology, unique monoflorals could be identified. Future studies like this may add market value to the honeys produced in unexplored natural areas in the CFR.

The specific composition of plant species, and therefore the potential for honey production at a geographic location, is influenced by many factors such as climate, soil type, topography, plant-pollinator relationships and anthropogenic influence including climate change, habitat destruction, frequency of fires, etc. (Crane 1975c; John et al. 2007; Nyunza 2018). Differences in honey composition between years or seasons of harvest within the same geographic location are mainly due to differences in nectar availability, which can be due to weather patterns, but can also be influenced by acute events such as fires and land-use change over time (Ogilvie and Forrest 2017; Phillips et al. 2018). Differences in individual colony nectar source preference is another possible reason for differences between honeys produced in similar geographic locations (Beekman et al. 2004). However, if honey from multiple colonies from the same apiary site is harvested and treated as a combined sample (as is the case with some samples in this study), the combined sample should reflect the overall resource availability and use across the landscape and therefore individual colony preference would not contribute to differences seen in honey samples.

The bee-plant community compositions differed across the apiary sites in this study, yet the botanical composition of the honeys produced from these sites were largely similar. The only exceptions were honeys from Boplaas and Kersefontein 2 and Boplaas and Thali Thali differing from each other. This shows that, even with high plant species diversity and different combinations of plant species available to honey bees, the bees still preferred foraging on certain plant species and utilised similar resources across space. This agreed with results of de Vere et al. (2017) who found that honey bees had specific foraging preferences and of the more than 400 genera of plants available to bees within the diverse National Botanic Garden of Wales, only 11% of these plants had pollen present in the honey samples collected. In their study, the authors also found no significant relationship between the area where plant species occurred and the amount of pollen present in the honey samples, indicating that honey bees show distinct preferences for specific plant species, irrespective of where the hives are placed. One reason for this might be differences in nectar quality between plant species, with bees generally preferring diverse sugar compositions and a total sugar concentration between 30 and 55% (Maurizio 1975a; Johannsmeier 2001b).

Looking at temporal differences in honey composition, the botanical origin of honeys from certain sites only differed between 2016 and 2017. This is likely due to differences in flower availability due to changes in climatic variables, particularly rainfall, between the two years. The average amount of rainfall recorded at three weather stations closest to the six apiary sites measured 260 mm in 2016 and only 169 mm in 2017 (South African Weather Service). This difference between the precipitation of the two years caused a visible change in the number of flowers produced by certain species (personal observation), and likely also in flowering phenology and amount of nectar produced in different species (Waser and Price 2016; Descamps et al. 2018; Phillips et al. 2018). This shows that bees will potentially shift their foraging preferences in the landscape when different resources are available in specific years and seasons.

The five most abundant plant families present in the honey samples from the West Coast were Fabaceae, Zygophyllaceae, Aizoaceae, Apiaceae and Asphodelaceae. The only other study published on the pollen composition of honeys in South Africa analysed 62 honey samples from seven vegetation types of South Africa, including eight honey samples from the sandveld area along the

West Coast. In that study the honey samples analysed consisted mostly of plants from the Aizoaceae (called Mesembryanthemaceae) and Fabaceae (called Papilionaceae) families (Johannsmeier 2001a). He also found one sample from a *Zygophyllum* species and proposed that the genus *Aspalathus* is probably important for honey production in the area too. Our findings support this, as we found *Z. morgsana* and *A. spinescens* (form A) pollen to be the two most common grains in the honey samples and two of the main monoflorals that were produced over multiple years and at more than one location. This indicates that nectar preferences of honey bees in this area have been constant over at least the past decade and a half.

One of the major limitations for palynology studies in South Africa, and Africa in general, is the lack of appropriate pollen libraries to use as reference material for pollen identification. This was illustrated by the Johannsmeier (2001) study where, even though some fynbos species were obtained to supplement his pollen library, most of the indigenous honey contributors in the area were still largely unidentifiable. Another major challenge of characterising the botanical composition of honeys in the CFR is the high biodiversity and large number of plant species found in any given area. Many species are closely related, making it difficult to distinguish between their pollen grains visually (Holt and Bebbington 2014). These challenges were largely overcome by generating subset pollen libraries that spatially and temporally correlated the specific honey samples to plants that could potentially have contributed to the samples. This demonstrates the importance of using field assessments of the flowering times and localities of different species in conjunction with melissopalynology to estimate the botanical composition of honey samples in areas of high floral diversity.

In the South African context, as well as in other developing countries where there are limited resources and funding for research, the classic palynology method is an inexpensive approach and indispensable in providing specific information about the relative contributions of different plant species to honey production. Unfortunately, both melissopalynology and modern DNA methods are both challenged by the over- and under-representation of pollen grains in honey from certain plant species. This challenge can be overcome for certain plant species through caged honey bee foraging experiments, where honey from a single plant species is produced under controlled conditions and the pollen contents of that honey variety then quantified (e.g. Demianowicz 1961, 1964). The honey produced exclusively from a single plant origin always delivers a relatively constant amount of pollen grains in the sample, known as the pollen coefficient (PC) for that plant species (measured in 1000's per 10 g of honey). When a honey sample containing pollen from multiple plant species is analysed using melissopalynology, the absolute percentages obtained for each pollen type can be corrected using the PC value (expected amount of pollen per 10 g) of each plant species. This reveals the relative quantities that each plant species' nectar actually contribute to the honey sample and new percentage contributions for each plant species can then be calculated (Sawyer 1988).

Bryant and Jones (2001) reviewed the literature regarding the historical development of PC values, as well as flaws in the methodology of developing PC values, and suggested that new and standardised caged experiments be conducted to obtain updated and more precise PC values for the nectar sources of premium honeys produced today. Unfortunately, PC values are unavailable for honeys produced from uncommon plant species for which there are no caged experiments and therefore the under- and over-representation of pollen in honey samples cannot be determined. In our study for example, foraging observations on species in the family Proteaceae would suggest that their nectar contribution to the honeys sampled should be much higher than what was identified by the pollen counts. Johannsmeier (2001a) also mentioned that the pollen counts for Proteaceae were unexpectedly low and were extremely under-represented in honey samples. Therefore, it is possible that monofloral samples produced from these species are undetected due to low pollen counts. On the other hand, the monofloral samples that were identified could contain over-represented pollen grains, which inflates the pollen counts to proportions higher than the actual nectar contributions from the plants. Rodopoulou et al. (2018) found that the identification of honey origins with under- or over-represented pollen grains could be verified when investigating additional factors such as the physicochemical composition of honeys. In Chapter 3, some physicochemical properties of the monofloral honeys identified here are investigated further to verify their pollen percentages.

Conclusion

Pollen identification is a crucial element in upholding the standards of honey products on the global market and plays a significant role in validating claims of the uniqueness and the origin of different honeys. The monofloral varieties identified in this study could potentially be marketed as exclusive honeys from distinct West Coast vegetation, with their uniqueness adding market value to the products and potentially boosting the beekeeping sector and income of local beekeepers. Even though the West Coast area has high plant diversity, honey bees produced key monofloral honeys from a few selected plant species. Therefore, even with the plant composition of the sites differing significantly from each other, honey bees prefer and utilise similar resources across space and time. This foraging preference ideally feeds into the optimisation of honey production, by targeting specific sites within the area where preferred bee-plants occur. Targeting sites, especially abundant in *A. spinescens* (form A), will also boost the production of monofloral honeys. Through the identification of the floral origin of unique honeys and promoting batch specific honey harvesting, it could be possible to promote the local beekeeping sector through improved origin-based marketing strategies.

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Chapter 3. The physicochemical properties of selected West Coast honeys and how these properties change with honey age.

Introduction

Honey is a complex, carbohydrate-rich food source produced by honey bees. The exact physical properties and chemical composition of honey are influenced by many factors, the most important of which is the origin and composition of the nectar source utilised by the bees to make the honey (Crane 1975; Persano Oddo and Piro 2004). Physicochemical properties can also naturally change as honey ages over time, or unnaturally through post-harvest procedures like excessive heating during the packaging process (Fallico et al. 2009; De-Melo et al. 2017). External influences, such as weather and climate, and the enzymes added by the honey bees themselves during the honey making process, also affect the physicochemical properties of honey, but these are less important than specific botanical origin in contributing to the unique characteristics of different honey varieties (White 1975a; Bogdanov 2011a). In general, honeys with similar botanical origins have more similar physicochemical compositions than honeys produced from different floral sources (White et al. 1962; Lazarevic et al. 2012; Jovetić et al 2017).

The specific physicochemical properties of honey directly influence its organoleptic characteristics relating to taste, odour and colour, which can vary greatly between honeys from different botanical origins (Crane et al. 1984; Bogdanov et al. 2004). Various chemical compounds contribute to the taste and aroma of honey, including different combinations of sugars, amino acids, polyphenols such as flavonoids and tannins, and volatile organic compounds such as carbonyls and alcohols (White 1975a; Manyi-Loh et al. 2011). The taste and colour of honey has major implications for labelling, marketing and selling the product. In terms of colour for example, lighter honeys are generally more expensive and sought after internationally, whereas darker honeys are more popular with consumers in certain European countries (Bogdanov et al. 2004; Belay et al. 2015). Therefore, the physicochemical properties of honey can be a useful way to characterise the product and to identify the appropriate market for it. However, measuring some of these physicochemical properties of honey can also reveal a lot about the quality, harvesting and processing history of a sample.

Honey is a supersaturated solution, consisting mainly of different carbohydrate sugars (60-85%), as well as some minerals, proteins, enzymes and amino acids, organic acids, phytochemical compounds, polyphenols and vitamins, concentrated in 12-23% water (White 1975a; Bogdanov et al. 2008; da Silva et al. 2016). Moisture or percentage water content of honey is dependent on many factors, including the degree to which the ripening process was completed in the hive, season of harvest and climatic conditions, and to a smaller extent the specific botanical origin of a honey (Bogdanov et al. 2004; De-Melo et al. 2017). Moisture is also an indication of a honey's ability to remain stable and resist spoilage by yeast fermentation. International regulations set a maximum limit for the moisture content of honey at 20% (Codex Alimentarius 2001). In general, any honeys with less than 17% moisture, regardless of yeast count, should be safe from fermentation (White 1975b; Bogdanov 2009). International regulations proclaim that the free acid content of honey samples should measure below 50 mmol/kg (Codex Alimentarius 2001). Any measurements above this threshold could indicate that unwanted fermentation of honey has occurred (Feás et al. 2010; Belay et al. 2013). The level and composition of the organic acid contents of honey also varies naturally because of differences in honey bee secretions, specific botanical origins of honey and environmental variables (De-Melo et al. 2017).

Most ripe honeys are dominated by the monosaccharides fructose (32-44%) and glucose (23-38%), making up anywhere between 75% and 95% of the total carbohydrate contents (White 1975a; Doner 1977; da Silva et al. 2016). Although many oligosaccharides have been identified in honey, disaccharides such as sucrose, maltose and turanose make up most of the remaining sugar composition of blossom honey (Bogdanov 2011a; De-Melo et al. 2017). The sugar composition of honey changes over time as it ripens within the hive. As water evaporates from the stored nectar, inverting enzymes such as invertase change disaccharides (sucrose) into monosaccharide building blocks (fructose and glucose). However, continued enzymatic reactions in honey's acidic environment also form new di- and trisaccharides in honey as it ripens and ages (White and Maher 1953; Ruiz-Matute et al. 2010). International regulations (Codex Alimentarius 2001) set out minimum requirements for the total amount of invert sugars (fructose plus glucose, 60%) as well as maximum limits for sucrose content of honeys (5%), as the levels of these sugars are a good indication of whether the product was suitably ripened before harvesting. The threshold for sucrose is also useful in revealing possible honey adulteration through the addition of cheap sweeteners to honey, or through the inappropriate feeding of sucrose syrups to honey bees (da Silva et al. 2016).

Environmental, geographic and botanical factors influence the mineral content of honey. The specific nectar used to produce the honey as well as the soil type in the area naturally affects the mineral content (Bogdanov et al. 2007; De-Melo et al. 2017), whereas some trace elements are also introduced to honey due to anthropogenic factors such as pollution and heavy metal contamination (Porrini et al. 2003; Solayman et al. 2016). Common minerals found naturally in honey include, among others, K, Na, Ca, Mg, Fe, Cu, P and S (White 1975a; Bogdanov et al. 2008). Trace element content of honey was previously measured as the amount of "ash", but nowadays total mineral content is determined by measuring the electrical conductivity of honey (Bogdanov et al. 2004). Honeydew honeys have a higher mineral content than honeys made from floral nectar, and the electrical conductivity standards set out in Codex Alimentarius (2001) are mainly for the purpose of distinguishing and classifying honey as either blossom (conductivity below 0.8 milli Siemens/cm) or honeydew honey (conductivity above 0.8 mS/cm).

Enzymes such as invertase that reduce the sugars in nectar are added to the honey by the bees when nectar is carried to the hive in their honey stomachs and regurgitated into the wax comb cells (Persano Oddo et al. 1999). Other notable enzymes in honey include glucose oxidase, which breaks down glucose into gluconic acid and hydrogen peroxide, and diastase (White 1975a; Bogdanov 2011a). Diastase is a starch digesting enzyme and, although it is thought to play a minor role in honey production, it is particularly useful when analysing the quality of honey (Bogdanov et al. 2004; De-Melo et al. 2017). Diastase activity decreases with honey age and has a high sensitivity to heat, which means it can be used to identify the excessive post-harvest heating of a honey product. The minimum requirement for diastase activity in honey set out by Codex Alimentarius (2001) is at least 8 DN (diastase number, also called DZ or Schade units) on the Gothe-Scale.

Another measure of honey freshness and treatment is the presence of hydroxymethylfurfural (HMF). Inversely correlated to diastase activity, HMF is an intermediate compound in the Maillard reaction and increases rapidly when glucose and fructose are degraded under prolonged high temperatures (Ajlouni and Sujirapinyokul 2010; Pasias et al. 2017). This often happens when honey producers heat up crystallised honeys to liquify their product for packing. Internationally the maximum limit for HMF content of honey samples is 40 mg/kg (Codex Alimentarius 2001). Diastase activity and HMF content are used in conjunction to determine honey freshness and the degree of honey processing (heating) after harvesting (Fallico et al. 2009; Pasias et al. 2017). All honeys intended for trade on local and international markets must meet the requirements set out by honey commissions and government legislation. In many countries, including South Africa, honey is exempt from the typical food labelling requirement of an expiry or best before date (e.g. South African Department of Agriculture 2000; New Zealand Food Safety 2018; National Honey Board, USA 2019). Despite this, it has been shown that the important properties relating to product quality may no longer meet the prescribed international limits after 6 to 20 months in storage (Cavia et al. 2007; Cavia et al. 2008; Fallico et al. 2009; Khalil et al. 2010).

South Africa is a country known for its rich floral biodiversity, especially the fynbos biome in the Cape Floristic Region (CFR) of the Western Cape Province. The CFR contains around 9000 plant species and is characterised by extremely high species endemism, with 70-80% of the species found nowhere else on earth (Rebelo et al 2006; Manning and Goldblatt 2012). Since the botanical composition of honey directly influences its physical and chemical properties, it is reasonable to expect that the high plant species richness found in the CFR could potentially lead to the production of honeys with unique physicochemical compositions. This has, however, not yet been adequately studied in South Africa (Anderson and Perold (1964); Table S2). Moreover, the physicochemical compositions of honeys are not routinely analysed by South African honey producers. Therefore, little information is available on the properties of honeys produced from indigenous vegetation. For South African honeys, the Codex standards (Codex Alimentarius 2001) as well as the legislation set out by the South African Department of Agriculture (DOA 2000) are followed when honeys are analysed. The DOA legislation is also largely based on the Codex standards, although there are subtle differences in the limits set out for each honey parameter (see Table 2). However, since research on local honeys are largely lacking, many beekeepers argue that the standards set out for local honeys should be re-evaluated to determine whether they are in fact valid for honeys produced from uniquely South African flora.

The main objective of this study was to investigate the different physicochemical properties of fresh and aged raw honeys produced from indigenous fynbos vegetation along the West Coast of South Africa. These data will shed light on the potential shelf life of CFR honeys, whether the current national regulations are relevant for raw honeys produced from fynbos vegetation, and whether local South African honeys comply with international regulations. This information could potentially boost the reputation of honeys from the West Coast region of South Africa on the local and international export market. Differences in honey physicochemical composition between apiary sites and production years are also assessed. Finally, as honeys produced from similar botanical sources should exhibit similar physicochemical compositions, the physicochemical properties of selected monofloral honey varieties are investigated in an attempt to verify their monofloral status as identified via their pollen estimates (Chapter 2).

Methods

Honey samples

Honey was harvested over a three-year period (2015 to 2017) from the beginning of September to the middle of December each year along the West Coast of South Africa. Due to an ongoing drought, honey sample sizes were low, particularly for the 2015 season, in which only three honey samples could be collected. The 2015 samples were therefore excluded from further physicochemical analyses. The following 63 raw honey samples were collected from six localities in 2016 (n = 33) and 2017 (n = 30) and analysed: Boplaas (n = 17), Middelkraal (n = 7), Kersefontein 1 (n = 10), Kersefontein 2 (n = 10), Thali Thali (n = 12) and Hopefield (n = 7). Melissopalynological analyses confirmed that all the honey samples from all the sites originated from indigenous flowering plants (Chapter 2). Seven monofloral varieties produced in more than one year or at multiple sites (i.e. $n \geq 2$) were identified: *Aspalathus spinescens* Thunb. (form A) (n = 13), *A. spinescens* (form B) (n = 3), *Aspalathus stricticlada* (R.Dahlgren) R.Dahlgren (n = 2), *Capnophyllum africanum* (L.) Gaertn. (n = 2), *Conicosia / Carpanthia* type (n = 6), *Lobostemon glaucophyllus* (Jacq.) H.Buek (n = 2) and *Zygophyllum morgsana* L. (n = 7).

Fresh honeys were stored in the dark at 4°C until the time of analysis. Of the 2016 fresh honey samples, 23 samples were randomly selected to test the effect of ageing on physicochemical properties. A subsample of each was placed in a new container and placed in a dark cupboard at room temperature for 12 months (23.32 ± 2.44°C, measured with an iButton data logger (Fairbridge Technologies, South Africa) every 30 minutes over an 11-month period) to be analysed as "aged".

Physicochemical analyses

All physicochemical analyses were performed on fresh (n = 63) as well as aged (n = 23) honey samples. Honey colour was measured using a Pfund honey colour portable photometer (Hanna Instruments, USA) with the resulting Pfund values (light transmittance displayed as a value between 0 and 150 in millimetres) corresponding to different honey colour categories (Table S3). The following physical and chemical parameters relating to the quality and unique characteristics of the honeys were analysed by Intertek Food Services (Bremen, Germany): 1) Sugar spectrum (fructose, glucose, sucrose, maltose and turanose content), 2) Moisture content, 3) Electrical conductivity, 4) Diastase activity, 5) Free acid content, 6) pH, 7) Hydroxymethylfurfural (HMF) content and 8) Polyphenol content (see Table 2 for units of measurement). The fresh honey samples from each year were sent for analyses directly after the respective fieldwork period ended.

Statistical analyses

The low sample sizes for specific mono- and multifloral honey varieties obtained prevented rigorous comparisons, requiring a more circumspect interpretation of the results. In Chapter 2, however, we showed differences between the botanical origin of honeys produced at the different apiary sites, as well as in the different years. Hence, location and year were used as a proxy for botanical composition in the following analyses. All significant differences are based on $p < 0.05$.

The overall physicochemical properties of fresh honey samples (n = 63) harvested over 2016 and 2017 at the different apiary sites were compared using multivariate analyses in PRIMER version 6 (Plymouth Routines in Multivariate Ecological Research, Plymouth Marine Laboratory, UK). The data for these analyses excluded Pfund colour measurement and total invert sugars, as these are correlated with other measured physicochemical variables. Data were "normalised" (i.e. the mean of a given physicochemical variable was subtracted from each datum entered for that variable and divided by the standard deviation of the given variable (Clarke and Gorley 2006)) and a resemblance matrix was generated using Euclidian distances. The overall physicochemical composition of honey samples was analysed with a Principal Components Analyses (PCA) on the normalised dataset. Vectors of physicochemical parameters were overlaid on the PCA if that variable had a Pearson's correlation of $r \geq 0.6$ to PC1 or PC2. A Permutational Multivariate Analysis of Variance (PERMANOVA), with apiary site included as a fixed factor (Boplaas = B, Middelkraal = M, Kersefontein 1 = K1, Kersefontein 2 = K2, Thali Thali = TT, Hopefield = H), year included as a random factor (2016, 2017) and a site x year interaction term, was also used to test differences in the overall physicochemical composition between sites and years, as well as within-site differences across years. Pair-wise t-tests were done to determine specific differences, if a factor was found to be significant in the model.

To examine the overall physicochemical composition of honeys before and after storage for 12 months ($n = 23$; fresh and aged), a PCA was run in PRIMER (Plymouth Routines in Multivariate Ecological Research, version 6: Plymouth Marine Laboratory, UK) on a normalised dataset for these samples. Vectors of physicochemical parameters were overlaid on the PCA if that variable had a Pearson's correlation of $r \geq 0.6$ to PC1 or PC2. The data for these analyses also excluded Pfund colour measurement and total invert sugars.

The effect of harvest year (2016 and 2017) on specific physicochemical parameters was compared with Mann-Whitney U tests, since all the data were non-parametric. The effect of harvest location (different apiary sites) on individual physicochemical parameters was tested using Kruskal-Wallis tests with multiple comparisons of the mean ranks of all groups, as all the data were non-parametric. Finally, to test the effect of storage time (fresh vs. aged) on specific univariate physicochemical parameters, t-tests for dependent samples were used for parametric variables and Wilcoxon matched pairs tests were used for non-parametric variables. All analyses were done in Statistica version 13.5 (TIBCO Software Inc., USA).

The monofloral honey varieties with the highest sample sizes (based on melissopalynological analyses (Chapter 1)) were *A. spinescens* (form A) ($n = 13$), *Conicosia / Carpanthia* type ($n = 6$) and *Z. morgsana* ($n = 7$). Multivariate analyses in PRIMER (Plymouth Routines in Multivariate Ecological Research, version 6: Plymouth Marine Laboratory, UK) was used to potentially verify the monofloral nature of these monofloral honey varieties. A PCA was run on a normalised physicochemical dataset for these samples. Vectors of physicochemical parameters were overlaid on the PCA if that variable had a Pearson's correlation of $r \geq 0.6$ to PC1 or PC2. The data for these analyses also excluded Pfund colour measurement and total invert sugars.

Results

Overall physicochemical composition

Fresh honey composition differed significantly between apiary sites (PseudoF = 4.13, df = 5, p = 0.001), with pair-wise comparisons illustrated in Table 1. A significant difference was also found between the physicochemical composition of honeys produced in the two harvest years (PseudoF = 7.37, df = 1, p = 0.001). Within site differences in overall honey physicochemical composition between the two harvest years (site x year interaction) were significant (PseudoF = 3.846, df = 5, p = 0.001) for honeys from Boplaas (t = 3.059, p = 0.002), Middelkraal (t = 2.55, p = 0.042), Kersefontein 2 (t = 2.024, p = 0.01) and Thali Thali (t = 2.25, p = 0.002). The honeys produced within a site were distinct across the two years (Figure 1), but low samples sizes for Middelkraal in 2017 (n=2) must be acknowledged.

Table 1: Pair-wise comparisons of overall physicochemical composition of honeys at the different apiary sites. Sites are Boplaas = B (n = 17), Middelkraal = M (n = 7), Kersefontein 1 = K1 (n = 10), Kersefontein 2 = K2 (n = 10), Thali Thali = TT (n = 12) and Hopefield = H (n = 7). Bold p-values indicate significance (p < 0.05).

Site	**B**	**M**	**K1**	**K2**	**TT**
M	0.497				
K1	**0.037**	0.182			
K2	**0.001**	**0.001**	**0.002**		
TT	**0.001**	**0.004**	0.065	**0.001**	
H	0.168	**0.034**	0.473	**0.001**	**0.023**

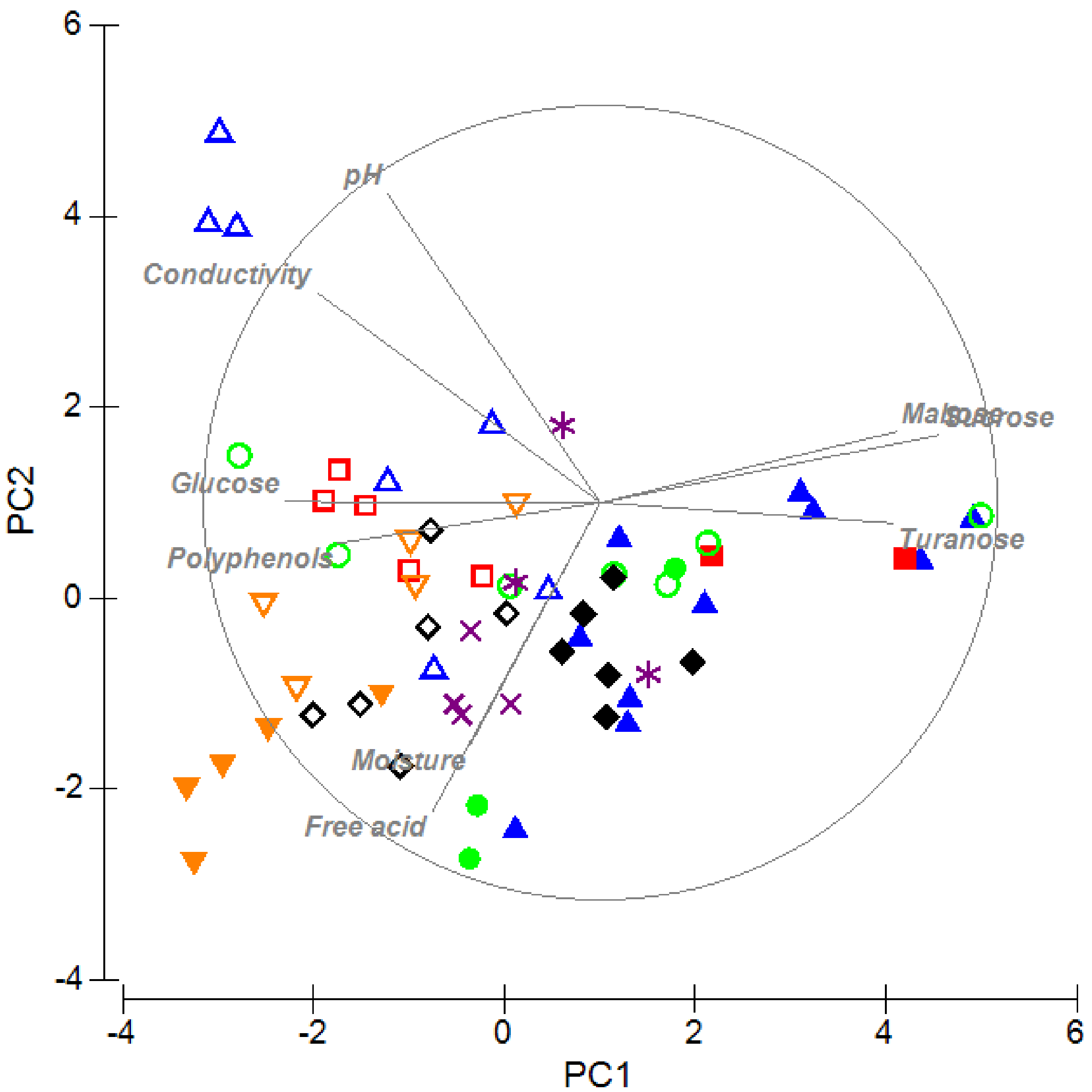

Figure 1: The physicochemical composition of honeys harvested across the two years within each apiary site along the West Coast. Sites are indicated as Boplaas = B (blue triangles), Middelkraal = M (red squares), Kersefontein 1 = K1 (green circles), Kersefontein 2 = K2 (orange inverted triangles), Thali Thali = TT (black diamonds) and Hopefield = H (purple symbols). Years are indicated as 2016 (open symbols and stars) and 2017 (closed symbols and crosses). Vectors of physicochemical properties with a Pearson's correlation of $r \geq 0.6$ to either PC1 or PC2 are included. The length of the vector indicates the importance of the parameter and the direction of the vector indicates the direction of its influence.

Individual parameters: compliance with standards

The median values obtained for each physicochemical parameter as well as the minimum and maximum values for each parameter is given in Table 2. Fresh honeys (n = 63) adhered to most of the standards set out by Codex Alimentarius (2001) and DOA (2000), except for sucrose (4 samples), moisture content (2 samples) and electrical conductivity (6 samples) falling outside the prescribed ranges for both standards; and invert sugar (only one sample) also fell outside the range set out by the DOA regulations. The subset of aged honeys (n = 23) also adhered to most of the Codex Alimentarius (2001) and DOA (2000) standards, except for electrical conductivity (4 samples).

Individual parameters: effect of location

Glucose content of honeys differed between apiary sites ($H_5 = 12.98$, $p = 0.024$), with honeys from Boplaas showing significantly lower values than honeys from Kersefontein 2 (Figure S1B). Samples from different locations also differed in turanose content ($H_5 = 21.27$, $p < 0.001$), with honeys from Kersefontein 2 having significantly lower turanose than honeys from Kersefontein 1 and Hopefield, and Thali Thali also showing lower measurements than Hopefield (Figure S1D). The final sugar measurement that showed significant differences between honeys from different sites was total invert sugars ($H_5 = 16.6$, $p = 0.005$), with Kersefontein 2 honeys having higher total invert sugars than Boplaas and Middelkraal (Figure S1F).

The electrical conductivity of honeys differed between sites ($H_5 = 13.23$, $p = 0.021$) with honeys from Boplaas having higher conductivity than honeys from Kersefontein 1 (Figure S2B). When comparing diastase activity between sites ($H_5 = 23.03$, $p < 0.001$), only Thali Thali honeys showed significantly lower activity relative to some other sites (Figure S2C). Honey free acids also differed between sites ($H_5 = 32.5$, $p < 0.001$), with Boplaas honeys having significantly lower concentrations than Kersefontein 2 and Thali Thali (Figure S2E). Pfund colour measurement of honeys ($H_5 = 35.83$, $p < 0.001$) as well as total polyphenol content of samples ($H_5 = 30.89$, $p < 0.001$) were different across the sites. Both Kersefontein sites were different from Boplaas honeys, which were significantly lighter with lower concentrations of polyphenols. Kersefontein 2 harvests also had overall darker colours and higher polyphenol measurements compared to honeys from Middelkraal (Figure S3).

Individual parameters: effect of year

Pfund honey colour was darker for honeys from 2016 compared to honeys from 2017 ($U = 180.5$, $p < 0.001$; Figure S4A) and similarly honeys harvested in 2016 had overall higher polyphenol content than honeys from 2017 ($U = 154.5$, $p < 0.001$; Figure S4B). When comparing honey sugars, glucose concentration ($U = 319.5$, $p = 0.016$) as well as total invert sugars ($U = 270.5$, $p = 0.002$) were higher in 2016 honey samples than in 2017 honeys (Figure S5). Honeys from 2016 also had higher electrical conductivity ($U = 160$, $p < 0.001$) as well as pH measurements ($U = 85$, $p < 0.001$) than honeys produced in 2017 (Figure S6).

Individual parameters: effect of storage

As samples aged, honey colour became darker ($Z = 4.07$, $p < 0.001$) and the polyphenol content also increased significantly ($t_{22}=4.71$, $p < 0.001$; Figure 2). Honey fructose content ($t_{22} = 5.27$, $p < 0.001$), glucose content ($t_{22} = 3.61$, $p = 0.002$), sucrose content ($Z = 3.72$, $p < 0.001$), maltose content ($Z = 2.76$, $p = 0.006$) as well as total invert sugars ($t_{22} = 4.3$, $p < 0.001$) decreased significantly over the 12-month storage period (Figure 3). The only sugar that increased with honey age was turanose ($Z = 4.2$, $p < 0.001$, Figure 3D). Other physicochemical parameters that increased as honey aged are HMF content ($t_{22} = -5.55$, $p < 0.001$, Figure 4D) and free acidity ($Z = 3.56$, $p < 0.001$; Figure 4E). Conversely, moisture content ($t_{22} = 4.55$, $p < 0.001$; Figure 4A), diastase activity ($t_{22} = 9.84$, $p < 0.001$; Figure 4C) and honey pH ($Z = 2.78$, $p = 0.005$; Figure 4F) decreased over the 12-month storage period.

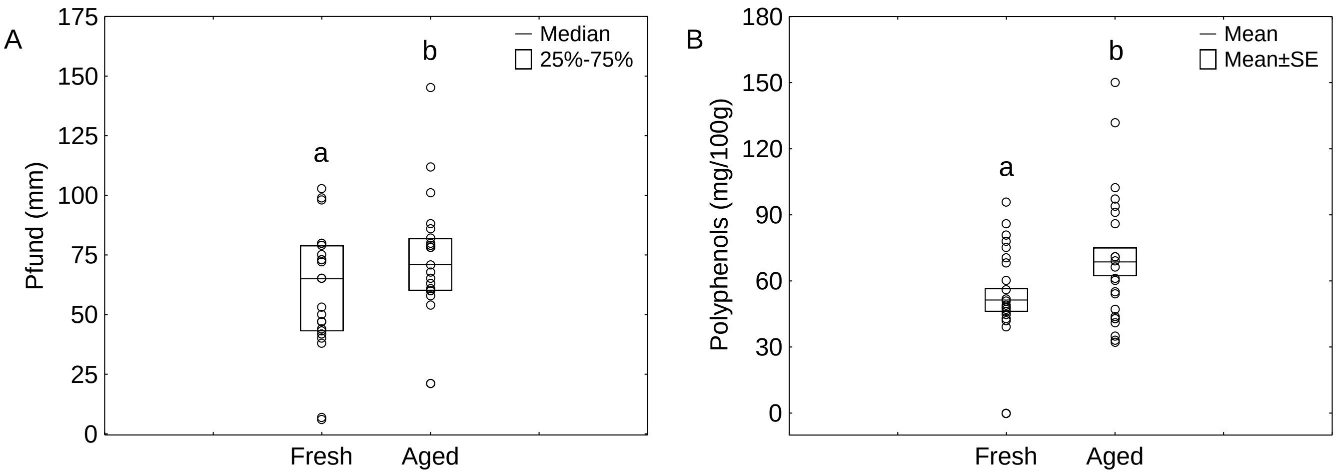

Figure 2: Honey colour (panel A) and polyphenol content (panel B) of fresh (n = 23) and aged honeys (n = 23) harvested along the West Coast. For colour and polyphenol content significant differences are indicated with different letters ($p < 0.05$), based on a Wilcoxon matched pairs test and a t-test for dependent samples, respectively.

Table 2: The physicochemical measurements of honey samples harvested along the West Coast of South Africa. The unit of measurement, as well as the official quality standards for blossom honey, are indicated in bold next to each physicochemical parameter. For the parameters where no official standard value is provided, general ranges are indicated as obtained from published literature (not in bold). The median, minimum and maximum values for each physicochemical parameter of fresh (n = 63) as well as aged (n = 23) West Coast honey is given and indicated in bold if it falls outside the permitted range of either Codex (2001) or South African Department of Agriculture (2000) standards.

		Standards		**Fresh honeys**			**Aged honeys**		
	Unit	**Codex**	**DOA**	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**
Fructose (F)	g/100 g	27.2 - 44.3 [1]		40.04	34.80	43.00	39.60	37.40	42.70
Glucose (G)	g/100 g	22.0 - 40.7 [1]		33.60	27.50	37.40	31.50	29.20	36.10
Invert sugars (F+G)	g/100 g	**> 60**	**> 65**	73.80	**62.90**	77.80	71.00	67.40	75.70
Sucrose	g/100 g	**< 5**	**< 5**	0.95	0.00	**9.40**	0.00	0.00	1.20
Turanose	g/100 g	n/a		1.20	0.00	2.00	2.10	1.60	2.70
Maltose	g/100 g	n/a		1.05	0.00	2.60	0.00	0.00	1.70
Moisture	%	**< 20**	**< 20**	16.70	14.60	**21.30**	15.80	14.70	18.80
Electrical conductivity	mS/cm	**< 0.8**		0.38	0.14	**1.04**	0.42	0.20	**1.11**
Diastase	DZ	**> 8**	**> 4**	23.15	10.50	59.70	18.90	6.80	28.10
HMF	mg/kg	**< 40**	**< 40**	0.70	0.10	3.10	5.90	0.00	17.50
Free acid	mmol/kg	**< 50**	**< 40**	21.35	9.20	39.50	22.50	12.30	32.10
pH	pH	3.4 - 6.1 [1]		4.00	3.70	5.90	4.10	3.80	5.60
Pfund	mm	0 - 150 [2]		52.50	2.00	112.00	71.00	21.00	145.00
Polyphenols	mg/100 g	5 - 1300 [3]		45.50	0.00	103.00	61.00	32.00	150.00

1. White et al. 1962; 2. Hannah Instruments, USA; 3. De-Melo et al. 2017

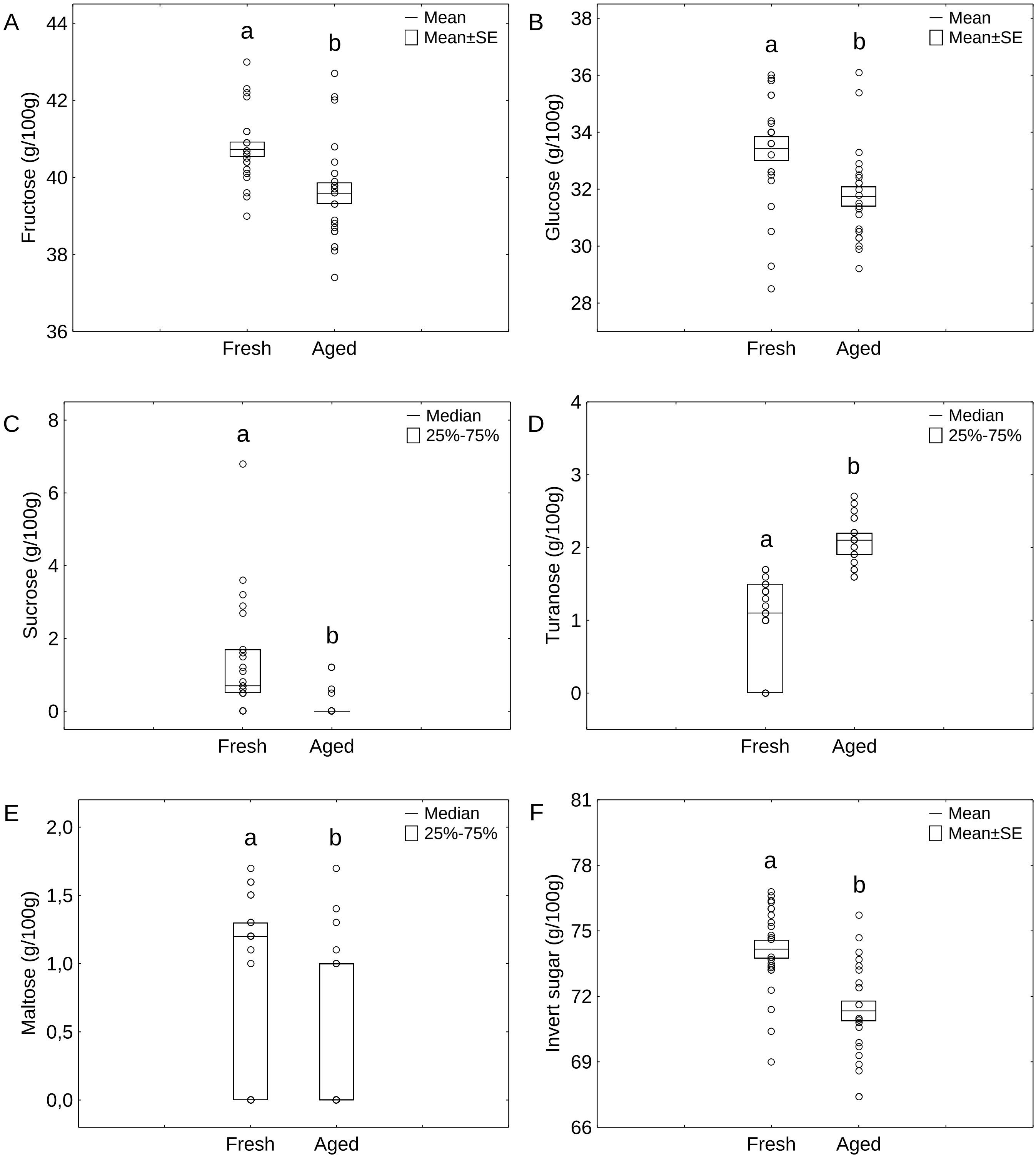

Figure 3: Sugar composition of fresh (n = 23) and aged honeys (n = 23) harvested along the West Coast. The different panels show: Fructose content (A), glucose content (B), sucrose content (C), turanose content (D), maltose content (E) and the total invert sugars (F) in grams per 100 g of honey. Significant differences based on either t-tests for dependent samples or Wilcoxon matched pairs tests are indicated with different letters ($p < 0.05$).

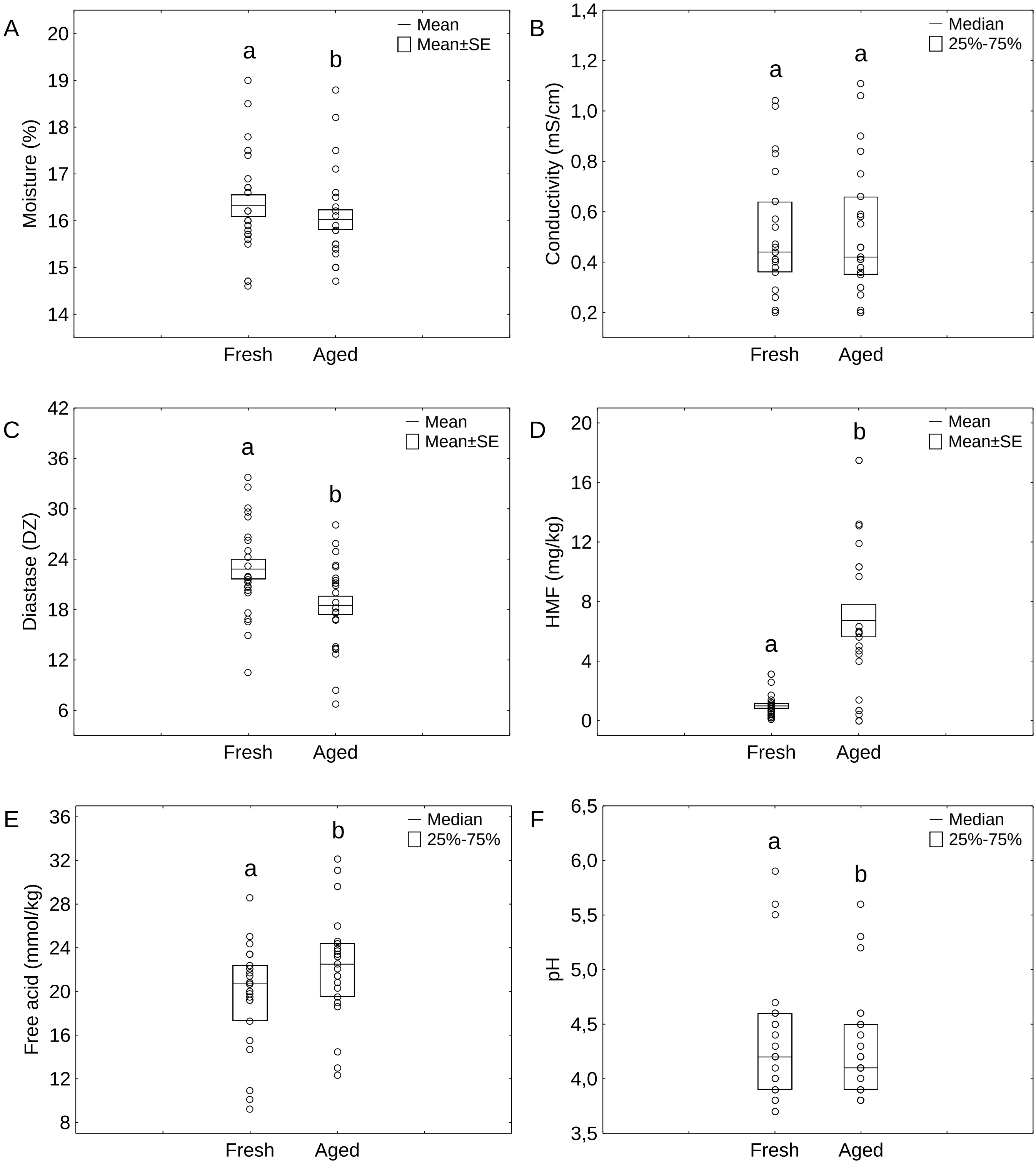

Figure 4: Physicochemical properties of fresh (n = 23) and aged honeys (n = 23) harvested along the West Coast. The different panels show: Moisture content (A), electrical conductivity (B), diastase activity (C), HMF content (D), free acid content (E) and pH (F). Significant differences based on either t-tests for dependent samples or Wilcoxon matched pairs tests are indicated with different letters ($p < 0.05$).

Monofloral honey varieties

The three main monofloral honey varieties harvested along the West Coast are distinct (Figure 5). Maltose, sucrose and turanose content differentiates *A. spinescens* (form A) samples, while electrical conductivity and pH are more characteristic of *Conicosia / Carpanthia* type. *Z. morgsana* samples cluster together based on free acidity, moisture and polyphenols.

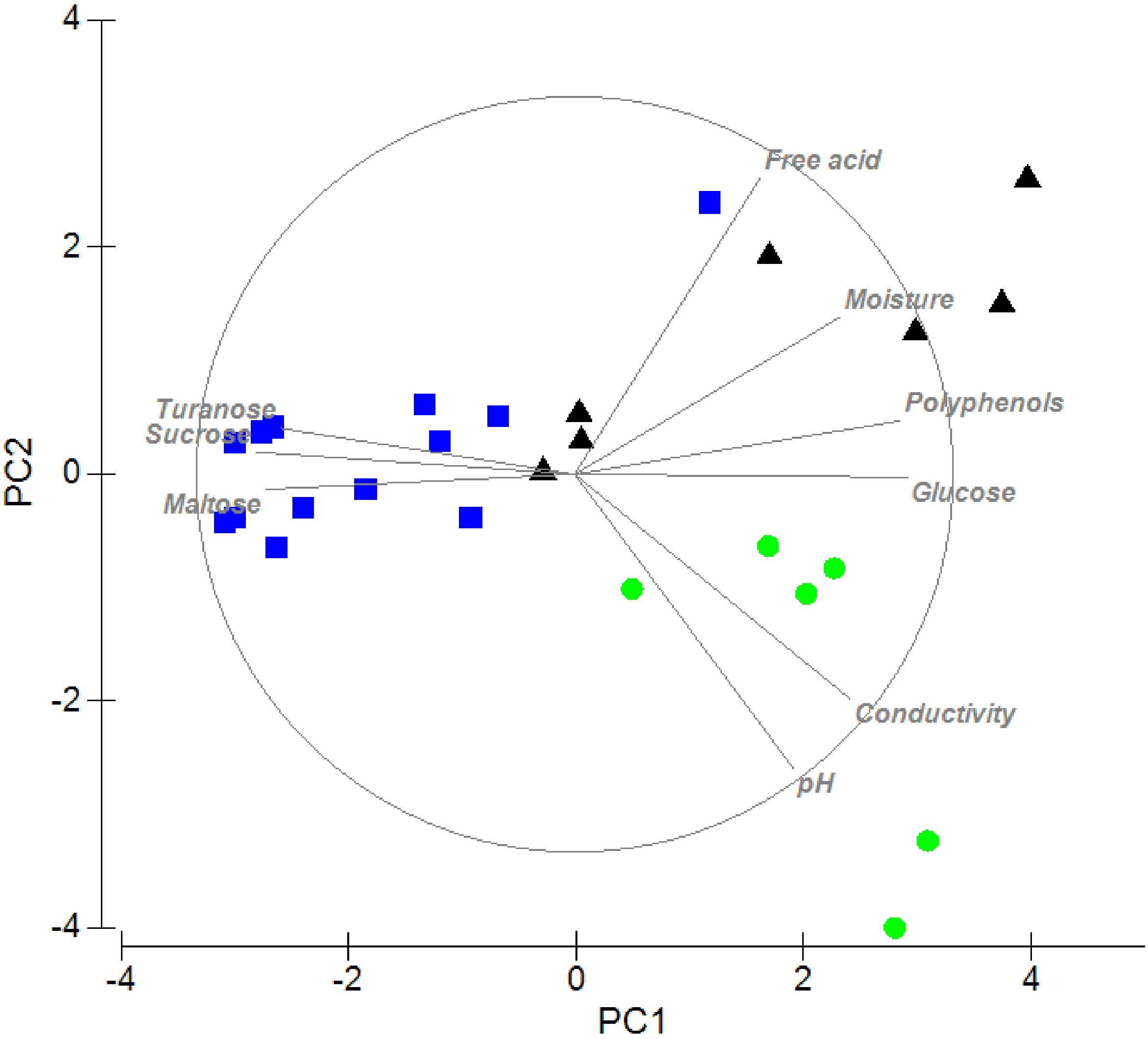

Figure 5: The physicochemical composition of monofloral honeys harvested along the West Coast. *Aspalathus spinescens* (form A) (blue squares; n = 13), *Conicosia / Carpanthia* type (green circles; n = 6) and *Zygophyllum morgsana* (black triangles; n = 7). Vectors of physicochemical properties with a Pearson's correlation of r ≥ 0.6 to either PC1 or PC2 are included. The length of the vector indicates the importance of the parameter and the direction of the vector indicates the direction of its influence.

Discussion

The composition of honeys harvested along the West Coast of South Africa varied in time and space. Honeys differed in their physicochemical properties between apiary sites as well as between harvest years. Here it was shown, like in previous studies, that the physicochemical properties of honeys vary spatially and temporally due to differences in honey botanical composition (e.g. White et al. 1962; El-Sohaimy et al. 2015; Warui et al. 2019). We revealed that the botanical composition of honey samples differed significantly between Boplaas and Kersefontein 2 (Chapter 2) and these botanical differences are here reflected in the physicochemical parameters (glucose content, total invert sugars, free acidity, honey colour and total polyphenols) that differ between the sites. Similarly, the botanical compositions of honey samples differed significantly between Boplaas and Thali Thali and this difference is most likely responsible for the differences seen in the physicochemical parameters between these sites.

Environmental fluctuations can cause variations in the floral resources available to honey bees across years and seasons (Waser and Price 2016; Descamps et al. 2018; Phillips et al. 2018). Consequently, honey physicochemical compositions will differ from one year to the next. The average amount of rainfall received in the study area in 2017 was only 65% of that received in 2016 (South African Weather Service). This difference in precipitation altered the availability of plant species between 2016 and 2017 (personal observation) and therefore could potentially explain the overall differences in physicochemical composition of honey samples between these two years. Furthermore, honey botanical composition differences found between the different years of honey production at Boplaas, Kersefontein 2 and Thali Thali (see Chapter 2) are potentially responsible for driving the differences seen in the physicochemical composition between years at these same sites.

It has been illustrated that multivariate analyses can be used to verify the botanical origins of monofloral honeys, as samples that are from the same botanical origin should have more similar physicochemical compositions and cluster together in multivariate space (e.g. Kukurová et al. 2008; Rodopoulou et al. 2018). White et al. (1962) and Jovetić et al (2017) also illustrated this when they found fewer differences in physicochemical composition when the botanical composition of honeys remained constant across space and time, for example when specific monofloral honey varieties from different locations or harvest seasons were compared. When the physicochemical compositions of the West Coast monofloral honeys were analysed they did separate out in multivariate space based on their botanical origins, but ideally many more samples from these varieties should be obtained to use this method of monofloral honey variety verification for South African honeys.

The Codex and DOA standards for honey specify two criteria for the sugar content of blossom honey: that the combined amount of fructose and glucose (total invert sugar) should be at least 60% (Codex) or 65% (DOA), and that the maximum amount of sucrose present in honey should be no more than 5% (DOA 2000; Codex Alimentarius 2001). Only one fresh honey sample out of 63 had lower than the allowed invert sugars prescribed by the DOA regulations. The measure of invert sugars indicates the ripeness of a honey, as the enzyme invertase breaks down the initial higher sucrose content of nectar into monosaccharides through the ripening process (Persano Oddo et al. 1999). If a honey sample has less invert sugars than allowed, it could potentially have been harvested when the honey was not yet ripe. However, since the one honey sample in question still adhered to the less strict Codex standards, it is safe to assume that is was ripe enough for harvesting. In this study, the temporal variation of total invert sugars is due to the differences in glucose content seen between apiary sites as well as years of production. As the amount of glucose in honey is related to the original sugar composition of the nectar collected by honey bees (Maurizio 1975; Doner 1977), differences in glucose content of honey could be due to the original nectar composition of the plants used to make the honeys differing spatially and temporally.

Four fresh honey samples also had higher than allowed sucrose content. The level of sucrose in honey is influenced by the botanical origin of the honey, but it can also be an indication that the honey ripening process was not completed before harvesting or that the bees were artificially fed with sucrose that ended up in the honey (De-Melo 2017). The latter is not applicable to the honey analysed in this study, as none of the hives at any of the sites were fed with sucrose during the honey harvesting period. Due to the natural areas where the hives were kept, the honey bees would also not have had access to alternative sources of artificial sucrose. The botanical origins of the samples with higher sucrose content were determined through melissopalynological analyses (Chapter 2) and it was found that three of those had similar botanical compositions, namely monofloral *A. spinescens* (form A) or sandbos honeys. Another monofloral sandbos sample from the 2015 harvest year, which was not included in this chapter's analyses, also showed higher than allowed sucrose, indicating that higher sucrose levels could be a trait associated with this specific botanical origin. More samples from this monofloral variety should be obtained to verify this hypothesis. Internationally, other honey varieties have been found to naturally have higher sucrose content than the 5% limit (e.g. alfalfa, citrus spp. and lavender) and therefore the sucrose limit for these honeys are more relaxed (Codex 2001). If it is found with continued testing that some fynbos honeys like sandbos indeed exhibit naturally higher sucrose content, exceptions should be made for these varieties when testing sugars.

The decrease in monosaccharide invert sugars and the disaccharide sucrose in honeys over the 12-month period supports the findings of previous studies focussed on the effect of honey ageing on physicochemical properties. Jiménez et al. (1994), White et al. (1962) and Castro-Vázquez et al. (2008), who studied honey samples from Spain, a variety of America honeys, and Spanish citrus honey, respectively, all found that fructose and glucose decreased with honey age. This decrease in monosaccharide building blocks happens naturally over time, because monosaccharides are utilised to build complex sugars through continued enzyme activity and acid reversion (White 1975a; Castro-Vázquez et al. 2008). Interestingly, some authors have found opposite trends in honey monosaccharides over time. In a study of Spanish honeys kept in the dark at room temperature, Cavia et al. (2002) showed increases in fructose and glucose content for most honeys. This suggests that in their study the disaccharides and complex sugars formed during honey storage were subsequently hydrolysed back into their monosaccharide building blocks.

Sucrose decreased significantly in honeys stored at room temperature. This decrease in sucrose is similar to findings by Jiménez et al. (1994) and Rybak-Chmielewska (2007), who studied honeys from Poland, and was expected due to the enzymatic reactions in the natural honey ripening process (De-Melo 2017). In contrast, White et al. (1962) and Castro-Vázquez et al. (2008) both found a small increase in sucrose content of American and Spanish honeys over their storage period. With regards to other disaccharides, we found a significant increase in turanose, but a decrease in maltose content over the 12-month ageing period. Changes in these sugars over time have also delivered mixed results in the honey literature. Similar to our results, Jiménez et al. (1994) found that turanose content in Spanish honeys increased with honey age and maltose decreased. In the study by White et al. (1962), they conversely found a large increase in maltose of American honeys. This may, however, be explained by their use of the term “maltose” to refer to all reducing disaccharides including, among others, turanose. Similarly, Kalimi and Sohonie (1964), in a study of Indian honeys, and Castro-Vázquez et al. (2008) found a general increase in higher sugars with aged honey, but in the latter study maltose showed the largest increase when stored at higher temperatures. Differences in turanose content found between honeys from different apiary sites are likely influenced by the original nectar composition of the honey due to floral availability at a particular site, as well as specific enzymatic activity during the honey ripening process.

The shelf life of a honey sample depends largely on its moisture and yeast content (White et al. 1962; Jiménez et al. 1994; Bogdanov 2011b). Overall, fresh honeys had good moisture contents, with only two samples out of 63 falling outside the prescribed limits of Codex Alimentarius (2001) and DOA (2000). Different factors influence the moisture content of honey, the most important of which is the degree to which the ripening process, i.e. the evaporation of water from stored nectar, has been completed in the hive by the time of harvest (Bogdanov et al. 2004; De-Melo et al. 2017). It is possible that frames from these two honey samples were not yet sufficiently capped before removal and extraction. For honey samples aged for 12 months, the moisture content significantly decreased with an average of 0.3% per sample. This decrease is minimal, but could potentially be explained by desorption, i.e. the loss of water to the air in the headspace of the jars. Desorption of moisture in honey takes place when honey is exposed to low relative humidity environments (Yao et al. 2003). Other authors either found no difference in honey moisture content between fresh and aged Spanish honey samples (Castro-Vázquez et al. 2008) or an increase in honey moisture over time, when Bangka rubber tree honeys were investigated (Evahelda et al. 2015). This is likely due to honeys absorbing moisture from the atmosphere in high humidity environments.

Electrical conductivity is directly related to the mineral content of a honey sample (Sancho et al. 1991; Feás et al. 2010) and due to the natural buffering action of minerals, this is also correlated to the honey pH (White et al. 1962). The electrical conductivity of honeys from different sites, as well as from different years of production, differed significantly. Besides potential differences in soil environmental minerals between sites, electrical conductivity also differs between different honey types (honey dew versus blossom honey) and is influenced by organic acids and proteins that vary greatly between honeys of different botanical origins (Terrab et al. 2003). No change in electrical conductivity was found in honeys stored for 12 months, which was to be expected as mineral elements do not degrade over time or during exposure to any external factors such as heat and light (da Silva et al. 2016). Six fresh honey samples had higher than allowed electrical conductivity and four aged samples also fell outside the prescribed limits for blossom honey. This may suggest the presence of honeydew elements in these honeys, although evidence of honeydew honeys being produced in South Africa is limited. Alternatively, these honeys could simply have a naturally high mineral content due to environmental factors.

Free acidity and pH are not correlated in honey. Usually pH remains constant during honey storage (White et al. 1962; Jiménez et al. 1994; Cavia et al. 2002), whereas free acidity increases over time (Bath and Singh 1999; Cavia et al. 2007). We found that pH actually decreased during honey storage at room temperature. This is similar to what Cavia et al. (2007) and Bath and Singh (1999) found, who studied Spanish honey samples and *Helianthus* and *Eucalyptus* honeys, respectively. We also found that free acidity, conversely, increased over time. White et al. (1962) ascribed this increase in free acidity over time to the continued enzymatic activity of diastase. Free acidity measurements above the 50 mmol/kg standard for honey could indicate that a honey has fermented, and the alcohol produced has been converted to acetic acid. The free acidity of honeys aged for 12 months remained within the prescribed limits. The free acidity of fresh honey differed between apiary sites and the pH differed between years of honey production. These differences could be due to variation in floral sources available at the different geographic locations during the two harvest years (De-Melo et al. 2017).

The two measures of honey freshness – diastase activity and HMF content – followed the expected trends of ageing honey (White et al. 1962; Castro-Vázquez et al. 2008; Cavia et al. 2008; Khalil et al. 2010). Diastase activity decreased significantly during storage and HMF increased. Neither variables were outside the prescribed limits for honey in fresh samples, nor after the 12-month ageing period. Diastase content did differ between honeys produced at different apiary sites. Even though diastase is an enzyme added by the bees during the honey making process, it can still vary between honeys from different geographic and botanical origins due to differences in nectar flow rates and physiological differences in honey bees (Bogdanov 2004).

Colour and polyphenol content of honey are correlated. Dark honeys generally contain more phenolic compounds and minerals than light honeys (White et al. 1962; Amiot et al. 1989; Bogdanov et al. 2007). The initial colour of honey is influenced by the botanical composition of the sample (Gonzales et al. 1999; Piotraszewska-Pająk and Gliszczyńska-Świgło 2015) and this was evident in our study, with varied honey colours produced at different apiary sites and across years reflecting different floral availability. Honeys also become darker with age or excessive heating due to Maillard reactions (da Silva et al. 2016; De-Melo et al. 2017). An increase in Pfund honey colour was found over the 12-month period, along with an increase in polyphenol content. Polyphenols are plant-derived secondary metabolites and their contents in honey are determined by the chemical composition of the nectar collected by honeys bees. Therefore, the botanical composition of the honey sample influences the polyphenol content, and explains the differences found in polyphenols between apiary sites and harvest years. Increased polyphenol content over time has not been reported elsewhere in the literature, but Brudzynski et al. (2013) did find that polyphenols in honey can be oxidized to form quinones, which in turn can form high molecular weight protein-polyphenol complexes. Whether this increased molecular weight could influence the way that polyphenols are measured is debatable.

Very little has been published on the physicochemical properties of South African honeys. The only data available is from Anderson and Perold (1964) who investigated, among other factors, the sugar composition, moisture content, ash content, pH, mineral content and colour of 66 honey samples from various origins (Table S2). Honeys sampled in this study had higher sugar components on average (fructose, glucose, invert sugars ad sucrose) compared to those in the study by Anderson and Perold (1964). They indicated a higher value for “maltose” compared to our findings, but in their study the term maltose was used to refer to all reducing disaccharides. They too had some samples outside the allowed Codex Alimentarius and DOA ranges for total invert sugars, sucrose content and total ash content. Even though it is not possible to assess the exact number of honeys from their 66 samples that did not comply with regulations, the authors concluded that the majority of honeys analysed compared favourably to honeys from America and that the American standards could easily be applicable to South African honeys. Similarly, our recommendation with regards to honeys standards is that both the current international as well as South African legislation is adequate for testing the quality of local raw honeys from the West Coast.

Conclusion

Here we show how specific physicochemical properties of raw CFR honeys harvested along the West Coast of South Africa differ over space and time and with honey age. The majority of raw honeys produced along the West Coast of South Africa complied with local and international regulations regarding physicochemical composition. The individual honeys that did not meet national or international standards on certain parameters probably deviated due to their botanical composition and geographic origin. Thus, botanical composition and geographic origin must be considered when assessing the results of physicochemical testing. Based on this, we propose that some exceptions regarding sucrose level should be made for certain honeys, for example *Aspalathus* honeys, but suggest that further study is required to substantiate this. For the most part, storing honey in the dark at room temperature for 12 months resulted in little change to the physicochemical composition and important honey parameters measured were still within the legal limits. Our recommendation is that the current South African legislation remain unchanged. We further propose that honey packers can still bottle and sell one-year old honeys from the West Coast, since their properties should still be within the legal limits if stored properly. However, further research into the ageing of South African honeys is necessary.

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Chapter 4. The antibacterial activity of selected West Coast honeys against *Staphylococcus aureus* bacteria.

Introduction

Honey has been utilised for therapeutic purposes by many cultures throughout human history and in recent years the antimicrobial, antioxidant and anti-inflammatory effects of honey on various skin conditions, burns, wounds and ulcers have been widely recognized in the scientific literature (Bogdanov et al. 2008; Lee et al. 2011; Manyi-Loh et al. 2011; Israili 2014; Miguel et al. 2017). Honey is an ideal antiseptic for wound care, as it prevents the wound dressing from adhering to the skin, it keeps the area moist, which assists in the healing process, and it prevents wound infection by microorganisms (Lusby et al. 2002; Molan and Betts 2004; Blair and Carter 2005). All honeys have some form of antimicrobial activity, which is caused by different combinations of bactericidal factors and mechanisms. The level of activity depends largely on the botanical and geographical origin of the honey, as well as the bacterial species in question. It is further influenced by the concentration of honey used, storage conditions and the post-harvest processing of honey (Kwakman et al. 2010; Voidarou et al. 2011; Irish et al. 2011; Moussa et al. 2012; Elbanna et al. 2014).

The main physical and chemical properties that contribute to the antibacterial activity of honey are its high sugar concentration and low moisture content, which causes osmotic stress and death in microorganisms, as well as the natural accumulation of hydrogen peroxide (H_2O_2) in honey when it is diluted (Molan 1992a; Bang et al. 2003; Kwakman and Zaat 2012). The H_2O_2 in honey causes oxidative damage and DNA degradation in microorganisms (Brudzynski et al. 2011), but the level is sufficiently low not to cause tissue damage to wounds (Bang et al. 2003). Other chemical components besides H_2O_2 can also contribute to the therapeutic potential of honey, to various degrees. These include specific phenolic compounds, flavonoids and organic acids originating from plant nectar that contribute to the antioxidant capacity of honeys (Al-Mamary et al. 2002; Gheldof et al. 2002; Miguel et al. 2017). Another compound that has been found to contribute to the antibacterial properties of some honeys is bee defensin-1, a small peptide that is part of the honey bee immune system and can culminate in the honey (Kwakman et al. 2010; Kwakman and Zaat 2012).

The naturally high acidity and low pH of honey is also believed to contribute to its antimicrobial activity by forming an acidic environment, which is unfavourable for most wound microbes (Lusby et al. 2002; Mandal and Mandal 2011), although varying results have been found when this factor was investigated experimentally (Molan 1992a; Bogdanov 1997; Kwakman et al. 2010). Correlations between other physicochemical parameters (e.g. electrical conductivity, honey colour and hydroxymethylfurfural content) and honey antibacterial activity have also been reported (Kerkvliet 1996; Taormina et al. 2001; Al-Mamary et al. 2002; Laallam et al. 2015), but the empirical evidence to support the contribution of these factors to the antibacterial activity of honey is lacking. Instead, many studies have found a stronger link between the actual H_2O_2 content of honeys and the resulting antibacterial activity (Dustman 1979; Brudzynski 2006; Brudzynski et al. 2011; Bucekova et al. 2014; Bucekova et al. 2019).

When water is added to honey, the enzyme glucose oxidase (GOX, which is secreted into the honey by honey bees during the honey making process) converts the glucose in honey into gluconic acid and hydrogen peroxide (Equation 1.1) (White et al. 1963; Kwakman and Zaat 2012). The H_2O_2 content of different honeys can vary considerably. The levels of GOX present in a honey sample may vary depending on the age- and diet-related hypopharyngeal gland development of honey bees (Pernal and Currie 2000; Bucekova et al. 2014). Catalase is another enzyme present in honey, which naturally decomposes H_2O_2 (Equation 1.2). This enzyme originates from the nectar as well as the pollen grains present in honey, and levels will vary depending on the amount and botanical origin of pollen grains and nectar that make up a specific sample (Dustman 1979; Brudzynski et al. 2011). The net production of H_2O_2 in honey is thus dependent on a combination of the production action of GOX and the decomposing action of catalase (Kwakman and Zaat 2012; Strelec et al. 2018).

$$1)\quad C_6H_{12}O_6 + 2H_20 + O_2 \xrightarrow{\text{GOX}} C_6H_{12}O_7 + 2H_2O_2$$

$$2)\quad 2H_20_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Equation 1: 1) The enzyme glucose oxidase (GOX) converts the glucose ($C_6H_{12}O_6$) in honey into gluconic acid ($C_6H_{12}O_7$) and hydrogen peroxide (H_2O_2) when honey is diluted with water in the presence of oxygen. 2) The enzyme catalase converts the hydrogen peroxide (H_2O_2) present in diluted honey back into water and oxygen.

In most honeys H_2O_2 is the major contributor to the total antibacterial activity (TA) of a honey sample, and therefore TA is generally defined as consisting of a predominantly peroxide activity (PA) component plus an additional non-peroxide activity (NPA) component. The NPA component consists of any other antibacterial mechanisms that are not H_2O_2, for example specific phytochemical compounds contributing to the antibacterial activity of honey (Bogdanov 1983; Mandal and Mandal 2011). The most prominent and well-studied honey with predominantly NPA is manuka honey from New Zealand, which exhibits high antibacterial activity due to the natural occurrence of methylglyoxal (MGO) in the honey (Allen et al. 1991; Molan 1992a; Irish et al. 2011; Kato et al. 2014; Johnston et al. 2018). This chemical compound is formed when the carbohydrate dihydroxyacetone present in the floral nectar of the manuka tree, *Leptospermum scoparium* J.R. Forst. & G. Forst, is non-enzymatically converted to MGO over time (Adams et al. 2008; Kwakman and Zaat 2012; Carter et al. 2016).

Contrary to PA in honeys caused by the production of H_2O_2, NPA factors in honey are not sensitive to light or heating, nor the inactivity of enzymes with honey ageing (White et al. 1963; Bogdanov 1997; Chen et al. 2012). Manuka honey, in fact, increases its NPA over time as more MGO is formed from dihydroxyacetone as honey ages (Irish et al 2011; Cokcetin et al. 2016). Therefore, honeys like manuka that show NPA are favoured above PA honeys for medicinal use, as they maintain their effectiveness and stability regardless of age and processing. Manuka honey can also be used undiluted, thus delivering a more powerful antibacterial effect (Molan and Betts 2004). PA honeys, on the other hand, first need to be diluted for wound care, as GOX is virtually inactive in undiluted honeys (White et al. 1963). The NPA of manuka honey is expressed and marketed as the Unique Manuka Factor™ (UMF), which is standardized as the equivalent of phenol concentration that shows the same efficacy, e.g. UMF 10^+ manuka has at least the same non-peroxide antibacterial activity as a 10% phenol solution (Blair and Carter 2005; Kato et al. 2014). The higher the UMF value, the better the NPA of the manuka honey. A UMF value of more than 10, i.e. with a phenol equivalence value of at least 10%, is considered therapeutically useful (Bischofberger et al. 2011; Irish et al. 2011).

There are different ways to determine the antibacterial activity of honey *in vitro*, including agar well diffusion assays (Allen et al. 1991; Brady et al. 2004; Patton et al. 2006; Manyi-Loh et al. 2010; Zainol et al. 2013), disk diffusion assays (Tumin et al. 2005; Patton et al. 2006; Ndip et al. 2007; Kirkpatrick et al. 2017), agar and broth dilution assays (Cooper et al. 1999; Basson and Grobler 2008; Ndip et al. 2007; Tan et al. 2009; Khan et al. 2014) and microtiter plate assays (Brady et al. 2004; Patton et al. 2006; Manyi-Loh et al. 2010; Sherlock et al. 2010; Kirkpatrick et al. 2017). In a study by Osés et al. (2016) the antibacterial activity of 50 honey samples from Spain were assessed against a common human wound pathogen, *Staphylococcus aureus* Rosenbach, and they found that the best way for the initial screening of honeys was to identify potential superior honeys through the agar well diffusion method. In honey studies, this method is often used in conjunction with a phenol equivalence assay, which allows for the comparison of honey antibacterial activity with that of a specific concentration of phenol – a general antiseptic. In fact, using the agar well diffusion method with reference to phenol has now become the standard method for analysing the TA, PA and NPA of honeys (Allen et al. 1991; Brady et al. 2004; Sherlock et al. 2010; Irish et al. 2011; Zainol et al. 2013). This method was initially used to assign a UMF value to all manuka honey batches produced, but a standard curve correlating UMF to the MGO content of manuka honey has since been generated and routine analysis of manuka samples now occur through direct measurement of this chemical component (UMF Honey Association 2019).

In recent years, investigating the medicinal properties of honeys from different geographic and botanical origins has gained popularity, especially in the quest to find potential alternative medicines to combat increasingly antibiotic resistant microbes such as the notorious wound bacterium, *S. aureus* (Goldstein 2007; Kwakman et al. 2008; Blair et al. 2009; Sherlock et al. 2010). Medicinal properties credited to a honey are one of the main factors influencing people's willingness to pay more for a honey (Kowalczuk et al. 2017), with some consumers also using honey in natural home remedies (Parisius et al. 2014; Quandt et al. 2015). Therefore, identifying honeys with potentially therapeutically beneficial properties could increase the marketability of that honey, and help to support the local beekeeping industry. South Africa has a very high biodiversity and the Cape Floristic Region (CFR) in the Western Cape Province especially has a high percentage of fynbos plant species that are endemic to the area (Manning and Goldblatt 2012). However, the potential for honeys with good antibacterial activity to be produced from these unique fynbos plant species is relatively unexplored to date.

In this study we conducted the first phenol equivalent antibacterial assay to identify the TA, PA and NPA of South African honeys, specifically investigating the antibacterial potential of honeys produced from indigenous fynbos plants from the West Coast. Additionally, the effect of honey ageing on the antibacterial activity of selected West Coast honeys was determined. Any relationship between the NPA activity of honey samples and the physicochemical parameters (Chapter 3) will be investigated. The aim is to identify therapeutically useful honeys produced from local flora that may be used to better characterise and market unique West Coast honey products. This may help promote South African honeys locally and on the international honey market.

Methods

Honey samples

Honey was harvested over a three-year period from the beginning of September to the middle of December each year along the West Coast of South Africa. A total of 66 raw honey samples were collected in 2015 (n = 3), 2016 (n = 33) and 2017 (n = 30). The samples came from the following different apiary sites: Boplaas (n = 19), Middelkraal (n = 8), Kersefontein 1 (n = 10), Kersefontein 2 (n = 10), Thali Thali (n = 12) and Hopefield (n = 7). Melissopalynological analyses confirmed that all the honey samples from all the sites originated from indigenous flowering plants (Chapter 2).

Seven monofloral varieties produced in more than one year or at multiple sites (i.e. $n \geq 2$) were identified: *Aspalathus spinescens* Thunb. (form A) (n = 13), *A. spinescens* (form B) (n = 3), *Aspalathus stricticlada* (R.Dahlgren) R.Dahlgren (n = 2), *Capnophyllum africanum* (L.) Gaertn. (n = 2), *Conicosia / Carpanthia* type (n = 6), *Lobostemon glaucophyllus* (Jacq.) H.Buek (n = 2) and *Zygophyllum morgsana* L. (n = 7).

All fresh honey samples were stored in the dark at 4°C until the time of analysis. From the 2016 fresh honey samples, 23 samples were randomly selected to test the effect of honey age on antibacterial activity. Each sample was split into two containers, of which one was kept at 4°C in the dark to be analysed as “fresh”, while the other container was placed in a dark cupboard at room temperature for 12 months (23.32 ± 2.44°C, measured with an iButton data logger (Fairbridge Technologies, South Africa) every 30 minutes over an 11-month period) to be analysed as “aged”. Each honey sample was assayed at least twice and on separate days to account for any variability between plates and conditions in the laboratory on different days.

Phenol equivalence assay

All laboratory work was conducted in the Microbiology Department at Stellenbosch University in a Biosafety Level 2 laboratory. Honey samples were analysed using the standardized phenol equivalence agar well diffusion assay for investigating the TA, PA and NPA of honey, according to Irish et al. (2011), with methods first described by Allen et al. (1991).

A laboratory testing strain of *S. aureus* (ATCC 25923) was obtained from the Microbiology Department at Stellenbosch University and grown in nutrient broth (Merck Millipore, USA) for 18 hours at 37°C. The broth was adjusted to an absorbance of 0.5 at 540 mm using a spectrophotometer (Spectroquant Pharo 300, Merck Millipore, USA). For each plate to be tested, a volume of 150 ml nutrient agar (Merck Millipore, USA) was prepared and stored at 60°C (Memmert, Germany) overnight. The agar was allowed to cool to approximately 45°C before being seeded with 100 µl of the adjusted *S. aureus* culture. The seeded agar was poured into a 245 x 245 mm large square assay plate (Corning Inc., USA) on a level surface. Agar plates were allowed to solidify and cool to room temperature and were stored at 4°C for one hour prior to use.

A 25 x 25 mm grid pattern was used to cut 64 wells into the agar with an 8 mm diameter flame-sterilised corkborer. Each well was numbered and a randomise function in Excel (Microsoft, USA) was used to randomly assign honey samples, controls and phenol solutions to the different wells in duplicate. Ten honey samples were prepared fresh daily by weighing 10 g of each honey using an analytical balance (Radwag XA 110/Y, Radwag Balances and Scales, Poland) and dissolving it thoroughly in 10 ml autoclaved RO water using a spatula and benchtop vortex. For TA testing, 250 µl of each honey solution was mixed thoroughly with 250 µl autoclaved RO water (hereafter called H^+) using a benchtop vortex. For NPA testing, the H_2O_2 content of 250 µl of each honey solution was neutralised by mixing it with 250 µl of a 5600 U/ml catalase solution (Sigma-Aldrich, USA) made up with phosphate-buffered saline (H^-).

Comvita UMF 18^+ manuka honey with a known antibacterial activity of at least 18% phenol (Comvita, New Zealand) was used as a positive control. Manuka honey was prepared in the same way as other honey samples to test for TA (M^+) and NPA (M^-). To control for the effect of pH and osmolarity on the antibacterial activity of honeys, an artificial honey was made consisting of 39% w/v fructose, 31% w/v glucose, 8% w/v maltose, 3% w/v sucrose (Sigma-Aldrich, USA) and 19% w/v autoclaved RO water, adjusted to a pH of 3.8 using gluconic acid lactone (Sigma-Aldrich, USA) (Brady et al. 2004; Khan et al. 2014). The sugar solution was filtered using 0.2 µl Acrodisc syringe filters (Pall Corporation, USA), stored at 4°C and replaced every four weeks. The solution was prepared in the same way as other honey samples (S^+ and S^-). Negative controls of autoclaved RO water (N^+) and catalase solution (N^-) were also included in each plate. Phenol standard solutions of 2%, 3%, 4%, 5%, 6% and 7% were prepared using phenol crystals (Merck Millipore, USA) and autoclaved RO water. Solutions were replaced every 4 weeks and stored at 4°C.

A 100 µl aliquot of each honey sample (H^+ and H^- solutions), positive control (M^+ and M^-), sugar solution (S^+ and S^-), negative control (N^+ and N^-) and each phenol solution was placed in its randomly assigned duplicate wells of each assay plate. Plates were incubated at 37°C for 18 hours. Thereafter the zone of inhibition that formed around each well was measured in mm in two directions using digital Vernier callipers (Mitutoyo, Japan) over a laboratory light box (Figure 1). The mean diameter of the zone of inhibition around each well was calculated and squared to account for the fact that samples become more diluted as they diffuse out of the well, with the area increasing as a function of the square of the diameter. A standard curve was generated by plotting the phenol concentrations against the mean squared diameter of their inhibition zones. To account for the dilution and density of honey (based on a mean density of 1.35 g/ml) the mean squared diameter of their inhibition zones was multiplied by 4.69 (Irish et al. 2011). The phenol equivalent antibacterial activity (% (w/v)) of each honey sample was then calculated from the standard curve.

If the zones of inhibition around a well was completely clear of any bacterial growth, the inhibition type was labelled as "full" and if any colony growth was detected within a zone of inhibition, the inhibition type was labelled as "partial" inhibition.

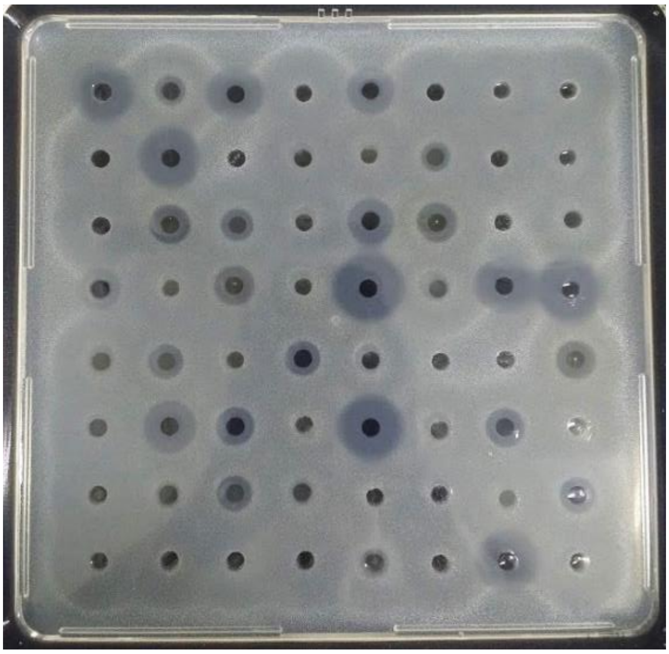

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Figure 1: An example of the large assay plates used for the phenol equivalence antibacterial assays, showing the inhibition zones formed around wells where honey samples and phenol standards inhibited the growth of *Staphylococcus aureus* bacteria.

Statistical analyses

The low sample sizes for specific mono- and multifloral honey varieties, based on the botanical origin of individual honey samples, obtained throughout this study prevented rigorous comparisons between locations and years. Despite this, we found differences between the botanical origins of honey produced at different apiary sites, and in different years (Chapter 2). Hence, location and year were used as a proxy for botanical composition in the subsequent analyses.

The phenol equivalent antibacterial activity of honey samples from the six apiary sites (Boplaas = B, Middelkraal = M, Kersefontein 1 = K1, Kersefontein 2 = K2, Thali Thali = TT, Hopefield = H) were compared using Kruskal-Wallis tests with multiple comparisons of the mean ranks of all groups. The antibacterial activity of honeys produced in the different harvest years (2016 and 2017) as well as differences in the antibacterial activity of honeys that showed partial and full inhibition were analysed with Mann-Whitney U tests. Due to an ongoing drought, only three honey samples were harvested during 2015, and thus this year was excluded from between-year comparisons. Finally, the effect of storage time (fresh vs. aged) on the antibacterial activity of honey was investigated with a Wilcoxon matched pairs test. These analyses were performed in Statistica version 13.5 (TIBCO Software Inc., USA). All significant differences are based on $p < 0.05$. Means with standard errors, medians and ranges are reported throughout.

Results

Assay controls

The average TA measured for Comvita UMF 18^+ over all the plates assessed in this study was 17.34 ± 0.09% phenol equivalence (median: 17.42; range: 16.02 – 18.88) and the average NPA was 17.17 ± 0.10% phenol equivalence (median: 17.33; range: 16.04 – 18.61). The daily variation in activity between manuka honey samples was within ± 2% of the indicated 18% phenol equivalence indicated by UMF 18^+ (Irish et al. 2011). Both negative controls (N^+ and N^-) had undetectable zones of inhibition in all the plates tested. Similarly, the artificial sugar solution (S^+ and S^-) also had no inhibitory effect on the bacterial growth of any plates measured.

Antibacterial activity of honey samples

None of the honeys tested had any detectable antibacterial activity when their NPA was analysed. Therefore, the total phenol equivalent antibacterial activity of West Coast honeys consists primarily of PA. All the antibacterial activity reported hereafter therefore refers to the TA of honey samples.

The antibacterial activity of honeys is presented according to Irish et al. (2011), who divided samples into 4 categories: 1) undetectable activity (< 5% phenol equivalence), 2) low activity (5-10% phenol equivalence), 3) potentially therapeutically beneficial activity (10-20% phenol equivalence) and 4) high activity (> 20% phenol equivalence).

Fifteen honey samples had antibacterial activity below the detection limit of the assay (which was approximately 5% phenol equivalence) and are indicated as < 5% in Figure 2. The average phenol equivalent antibacterial activity of all honey samples was 11.86 ± 0.56% (median: 12.70; range: < 5 – 23.44) and the average variation in activity within individual honey samples tested on different days was 1.45%. Detectable activity was found in the remaining 51 samples, with a mean activity of 13.81 ± 0.44% phenol equivalence (median: 13.66; range: 6.49 – 23.44). Forty-six samples (69.70% of all honeys) had a phenol equivalent antibacterial activity above 10% and therefore can be considered potentially therapeutically useful. Two samples showed antibacterial activity above 20% phenol equivalence (Figure 2).

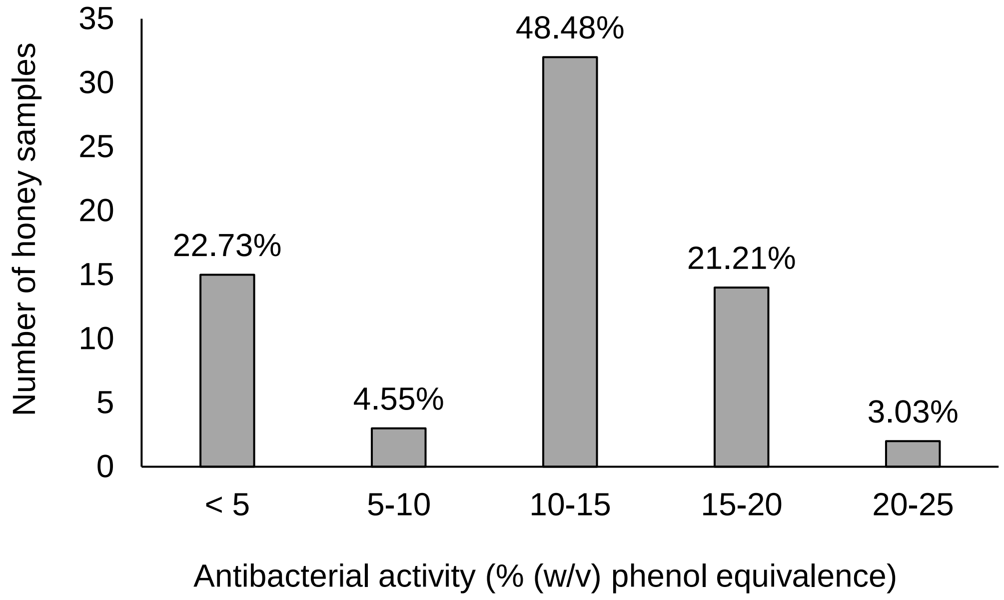

Figure 2: The total phenol equivalent antibacterial activity of West Coast honey samples (n = 66). Honey samples are divided into five activity brackets of 5% each, spanning the four antibacterial activity categories: 1) undetectable activity (< 5% phenol equivalence), 2) low activity (5-10% phenol equivalence), 3) potentially therapeutically beneficial activity (10-20% phenol equivalence) and 4) high activity (> 20% phenol equivalence).

The antibacterial activity of honey differed significantly between the six apiary sites ($H_5 = 18.93$, $p = 0.002$), with honeys from Thali Thali showing significantly lower activity than those from Hopefield and Middelkraal (Figure 3A). Thali Thali was the site with the overall lowest average antibacterial activity of 7.60 ± 0.82 % (median: < 5; range: < 5 – 12.08), whereas Hopefield was the site with the highest average activity at 14.79 ± 1.58% (median: 16.52; range: < 5 – 17.96). Honey harvested in 2016 had significantly higher phenol equivalent antibacterial activities than honeys harvested in 2017 ($Z = 2.09$, $p = 0.0367$) (Figure 3B), with honeys from 2016 having an average antibacterial activity of 12.85 ± 0.92% (median: 13.73; range: < 5 – 23.44) and 2017 showing an average activity of 10.96 ± 0.68% (median: 11.78; range: < 5 – 17.67). Honeys with full inhibition had overall higher phenol equivalent antibacterial activities compared to honeys that exhibited partial inhibition ($Z = -3.67$, $p < 0.001$) (Figure 3C), with an average antibacterial activity of 15.46 ± 0.58% (median: 14.96; range: 10.85 – 23.44) versus 12.35 ± 0.51% (median: 12.27; range: 6.49 – 18.76) for samples with partial inhibition.

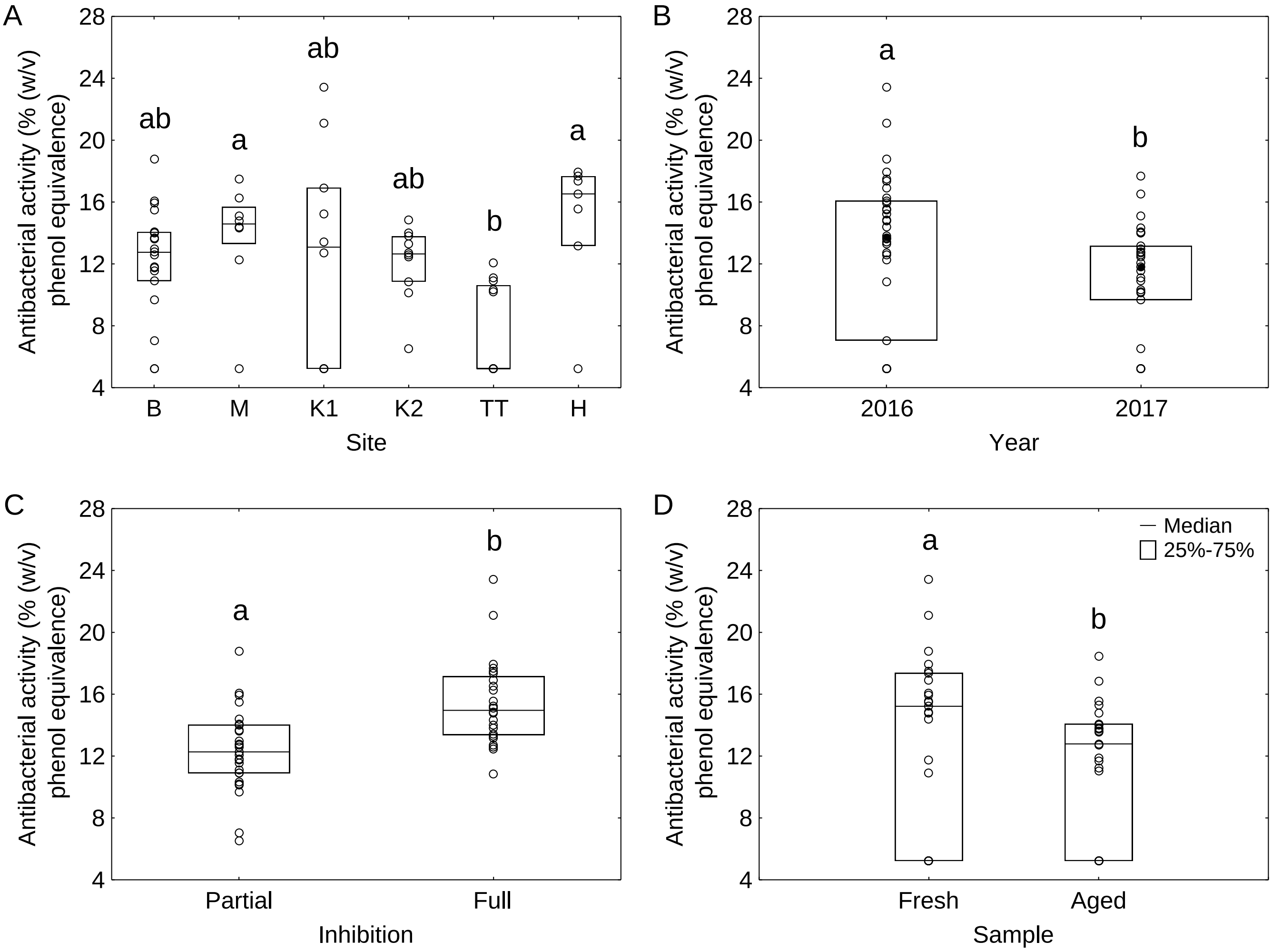

Figure 3: The phenol equivalent antibacterial activity of A) honey harvested at the six apiary sites along the West Coast: Boplaas = B (n = 19), Middelkraal = M (n = 8), Kersefontein 1 = K1 (n = 10), Kersefontein 2 = K2 (n = 10), Thali Thali = TT (n = 12) and Hopefield = H (n = 7); B) honey samples harvested in 2016 (n = 33) and 2017 (n = 30); C) honey showing partial (n = 27) and full (n = 24) inhibition against *Staphylococcus aureus* bacteria. D) Changes in the phenol equivalent antibacterial activity of honey after ageing for 12 months. Significant differences are indicated with different letters ($p < 0.05$).

Monofloral honey varieties

Out of the 66 honey samples analysed in this study, the monofloral *C. africanum* honeys had the highest phenol equivalent antibacterial activity with an average of 22.28% (Table 1; Figure 4). Of the seven monofloral varieties analysed, only the *C. africanum* and *A. spinescens* (form B) samples exhibited full bacterial growth inhibition in all of the samples tested. All the monofloral varieties had potentially therapeutically beneficial TA values of above 10% phenol equivalence, except for *A. stricticlada* (Table 1; Figure 4). However, due to low sample sizes and the large variation of antibacterial activity within honey types, differences between the monofloral varieties were not statistically significant (see Figure 4 for visual presentation only).

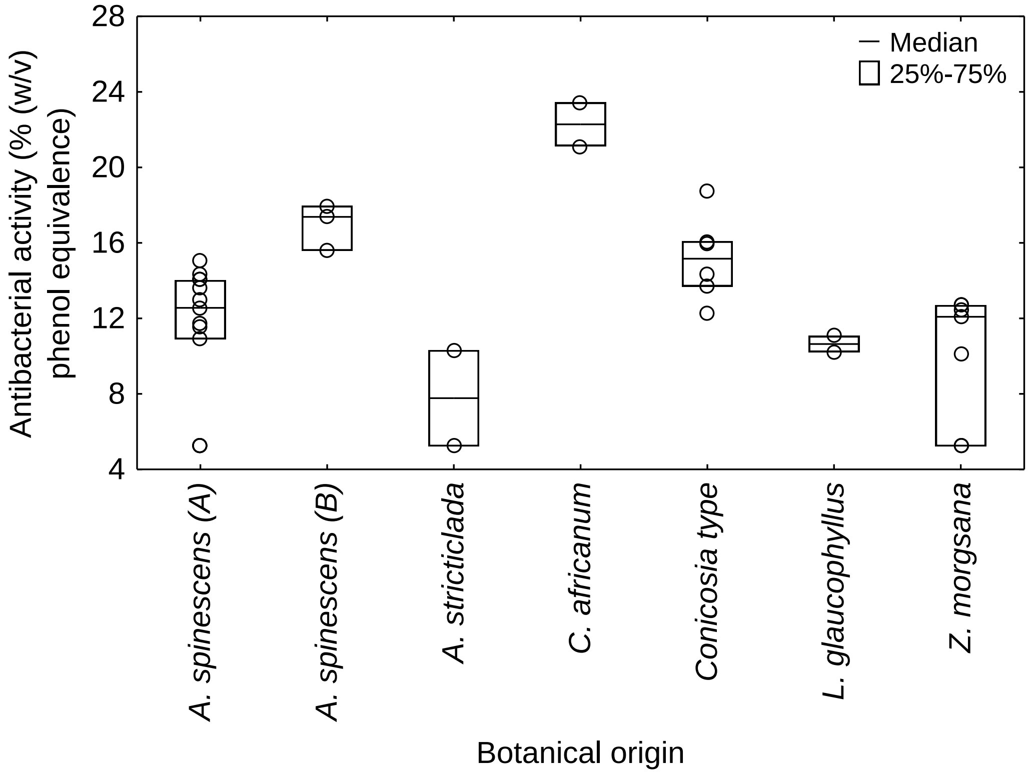

Figure 4: The phenol equivalent antibacterial activity of the seven monofloral honey varieties harvested along the West Coast of South Africa: *Aspalathus spinescens* (form A) (n = 13), *Aspalathus spinescens* (form B) (n = 3), *Aspalathus stricticlada* (n = 2), *Capnophyllum africanum* (n = 2), *Conicosia / Carpanthia* type (n = 6), *Lobostemon glaucophyllus* (n = 2), *Zygophyllum morgsana* (n = 7).

Effect of age on antibacterial activity

Phenol equivalent antibacterial activity decreased significantly as honey aged ($Z = 3.57$, $p < 0.001$). Fresh samples showed an average of 13.45 ± 1.17% (median: 1.15; range: < 5 – 23.44) phenol equivalent antibacterial activity and this decreased to 11.58 ± 0.9% (median: 0.88; range: < 5 – 18.45) after one year of ageing (Figure 3D). The average loss in antibacterial activity was 1.87% across all the samples tested, with a maximum decrease of 4.99%.

Table 1: The phenol equivalent antibacterial activity of the seven monofloral varieties harvested along the West Coast of South Africa. The number of samples, their average, median and range of total antibacterial activity (TA), as well as their type of inhibition against *Staphylococcus aureus* bacteria, are shown.

Plant species	Samples	Average TA (%)	Median TA (%)	TA range (%)	Samples with activity	Inhibition type (n)
Aspalathus spinescens (form A)	13	11.28	12.56	< 5 – 15.09	10	Partial (8); Full (2)
Aspalathus spinescens (form B)	3	16.97	17.38	15.58 – 17.96	3	Full (3)
Aspalathus stricticlada	2	7.77	7.77	< 5 – 10.33	1	Partial (1)
Capnophyllum africanum	2	22.28	22.28	21.13 – 23.44	2	Full (2)
Conicosia / Carpanthia type	6	15.19	15.16	12.27 – 18.76	6	Partial (6)
Lobostemon glaucophyllus	2	10.64	10.64	10.21 – 11.07	2	Partial (2)
Zygophyllum morgsana	7	10.08	12.08	< 5 – 12.76	5	Partial (4); Full (1)

Discussion

This is the first study to determine the phenol equivalent antibacterial activity of South African honeys. The antibacterial activity of West Coast honeys sampled is primarily due to the production of hydrogen peroxide in honey with no NPA detected in any of the samples. Almost 70% of fresh honey samples showed antibacterial activity above 10% phenol equivalence, which means that they are potentially therapeutically useful (Bischofberger et al. 2011; Irish et al. 2011). After ageing honeys for one year, the antibacterial activity decreased significantly, although the average antibacterial activity of samples tested remained above 10% phenol equivalence and was therefore still potentially therapeutically beneficial.

Other studies on the general antimicrobial activity of fynbos honeys has shown varied results. One such study was on the antifungal activity of South African honeys, conducted by Theunissen et al. (2001). Here one fynbos sample was investigated against the growth of *Candida albicans* (C.-P. Robin) Berkhout and did not show significant antimicrobial activity. Similarly, Basson and Grobler (2008) tested the antibacterial and antifungal activity of two fynbos honeys (one monofloral *Leucospermum* honey and one multifloral variety) against *C. albicans*, *Escherichia coli* (Migula), *S. aureus* and different *Streptococcus* strains. Neither of the two indigenous honey samples displayed high antimicrobial activity, and neither warranted medical grade status. In contrast, a study by Manyi-Loh et al. (2013), which investigated the activity of selected South African honeys, found that a multifloral honey sample containing fynbos species could potentially be used as an antibacterial agent against ailments caused by *Helicobacter* bacteria. The final study that investigated fynbos honey samples was by Khan et al. (2014) who tested the antibacterial properties of 10 honey samples labelled as fynbos against a wide variety of pathogens commonly associated with wound infections. They found that one particular fynbos sample, produced from *Erica* species, displayed broad spectrum antimicrobial activity. In all of these studies, the botanical origin of the honeys was identified by the beekeepers themselves and not through melissopalynology.

In this study, honeys from different apiary sites showed differences in their antibacterial activities, and honeys produced in the two main harvest years also had different efficacy against *S. aureus*. Other studies on the antibacterial activity of honey produced in different areas and from different floral sources have also found that botanical origin is an important determinant of antimicrobial activity in PA honeys (Elbanna et al. 2014; Strelec et al. 2018). Yet the antibacterial activity of honey from specific botanical sources and geographic areas can be very variable (Malika et al. 2004; Irish et al. 2011; Bucekova et al. 2014). This is in accordance with our findings, where the antibacterial activity of the monofloral samples investigated showed large variation within honey samples of the same variety. Therefore, it is generally accepted that floral source alone is an unreliable predictor of the antibacterial activity of honey (Irish et al. 2011; Chen et al. 2012). The predictability of antibacterial activity in honeys from specific botanical origins is complicated mainly because the final production of H_2O_2 in a honey sample is dependent on GOX as well as catalase activity in honey (Kwakman and Zaat 2012; Strelec et al. 2018).

Many studies have found a strong positive correlation between the actual H_2O_2 content of honeys and the resulting antibacterial activity (Dustman 1979; Brudzynski 2006; Brudzynski 2011; Bucekova et al. 2014; Bucekova et al. 2019), however there is not always a correlation between GOX content and H_2O_2 production in honey (Strelec et al. 2018; Bucekova et al. 2019). This indicates that catalase might actually be playing a significant role in determining the final peroxide antibacterial activity of honeys and because catalase originates from the nectar sources used to produce the honey, botanical composition does in fact influence PA in honey (Kwakman and Zaat 2012) – although not in a predictable manner. Therefore the differences in antibacterial activity found between apiary sites and between years in this study could at least partly be related to fluctuations in environmental variables that caused differences in the botanical composition of samples from different geographic areas as well as from years with different floral resource availability (Chapter 2).

Hydroxymethylfurfural (HMF) in honey is an indicator of honey freshness that increases with honey age and heating (Chapter 3). Similarly, H_2O_2 production in honey is also affected by post-harvest processing. The GOX in honey, and therefore the production of H_2O_2 and the resulting PA, is sensitive to heat and exposure to light, which causes the antibacterial activity to decrease as honey is exposed to these factors over time (Molan 1992b; Chen et al. 2012; Kwakman and Zaat 2012). With GOX in honey decreasing as honey ages, it makes sense that there would be a negative correlation between HMF content and the PA of honey samples, as these parameters are affected by the same processes. This correlation has not been widely reported in the literature (Kerkvliet 1996) and some studies found no relationship between HMF and antibacterial activity (Laallam et al. 2015). With more investigation into the relationship between HMF and antibacterial activity, this could potentially be used as a predictor of the PA potential of honeys. We also found that the antibacterial activity of honey did significantly decrease over a 12-month period. Similarly, Irish et al. (2011) and Elbanna et al. (2014) tested the effect of ageing on the antibacterial activity of honeys and found that the activity of samples showing PA decreased over time. Conversely there was no correlation between HMF and NPA in Australian honeys (Cokcetin et al. 2016), because MGO does not decrease with honey age.

Khan et al. (2014), who measured the antibacterial activity and pH of the South African honey samples, found pH to be between 3.89 and 5.09, postulating that this property of honey contributed to antibacterial activity. However, they did not account for pH in their assay controls nor specifically test whether pH is correlated to antibacterial activity. In our study controls, the pH adjusted sugar solution did not cause any inhibition of *S. aureus* growth. This agrees with results of Isla et al. (2011) and Bogdanov (1997), where the latter study rather showed a correlation between free and total acidity (see Chapter 3 for differences between pH and free acidity in honey samples).

Here we characterised the antibacterial activity of West Coast honey samples in relation to phenol but appreciate that future studies should focus on determining the minimum inhibitory concentration (MIC) of those honeys that showed high antibacterial activity. This is especially true for *C. africanum* monoculture honey, which had the highest antibacterial activity of all the honeys tested. Minimum inhibitory concentration assays could be done using a broth dilution method (Osés et al. 2016) and testing the antibacterial activity of the honey samples against a larger variety of microorganisms: other gram-positive bacteria, gram-negative bacteria and yeasts. Testing the antimicrobial activity of honey against *S. aureus* has become the standard protocol to test antimicrobial properties of honey, but it is not representative of the antimicrobial activity against other microorganisms (Kwakman and Zaat 2012). The major drawback of the agar well diffusion method used, is that the size of the inhibition zone depends on the rate at which the honey diffuses through the agar. If a honey has antibacterial activity due to compounds with a high molecular weight, it might produce a smaller zone and therefore be incorrectly assigned a low antibacterial efficacy (Kwakman and Zaat 2012).

In this study, we found that some honeys showed partial inhibition and not full inhibition of *S. aureus* growth, with partial inhibition samples on average being less antibacterial than full inhibition samples. This was not necessarily an obvious result, as honeys could theoretically show small zones of full inhibition or large zones of partial inhibition. This distinction in inhibition type and the acknowledgement of incomplete inhibition where honey only retards the growth of microorganisms instead of completely preventing it, has also been found in other studies (Theunissen et al. 2001; Aljadi and Yusoff 2003; Mulu et al. 2004; French et al. 2005; Mandal et al. 2010). Unfortunately, this phenomenon is potentially underreported in the literature, as many studies assess the zones of inhibition on a black background (e.g. Irish et al. 2011). This makes it difficult to see the growth of individual colonies within the zone, compared to assessment over a light box. Theunissen et al. (2001) specifically found that fynbos honey from South Africa only partially inhibited the growth of *C. albicans*. It is still unclear whether honeys that deliver partial inhibition are as effective in preventing infection, but further investigation of these honeys could perhaps deliver full inhibition when tested at a higher concentration. Honeys in the standard phenol equivalent antibacterial activity assay are routinely assessed at 25% of the full honey, yet the optimal honey concentrations for H_2O_2 production is between 30 and 50% (Bang et al. 2003).

Conclusion

The antibacterial activity of West Coast honeys can be attributed to the activity of hydrogen peroxide. Many of the samples tested showed potentially therapeutically useful antibacterial activity, although the variation in antibacterial activity of specific monofloral honey varieties was quite high. The monofloral variety, *C. africanum*, showed the highest antibacterial activity of all honeys tested. As the antibacterial activity of honey is highly variable between geographic and botanical origins, these results should be taken judiciously – especially due to the limited honey samples that were available for study. More honey samples from the West Coast must be tested using this phenol equivalence assay to more accurately determine the antibacterial potential of CFR honeys. The honey varieties that consistently show high antibacterial activity should also be tested in MIC assays against a variety of pathogens to further investigate their full therapeutic potential. Establishing the therapeutic potential of local honeys offers a marketing opportunity to promote medicinal honeys from the West Coast of South Africa.

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Chapter 5. Honey production along the West Coast: perspectives and suggestions for the South African beekeeping industry.

Honey production in a changing climate

The investigation of the botanical origin, physicochemical properties and antibacterial activity of honeys produced from indigenous Cape Floristic Region (CFR) vegetation along the West Coast of South Africa was conducted during the worst drought that the Western Cape Province has experienced in more than a century. The study period was characterised by 30-50% below average rainfall between 2015 and 2017 (Botai et al. 2017; Otto et al. 2018). The impact of this drought on the agricultural sector was severe, with related losses in crop and livestock production negatively affecting the economy (Ntombela 2017). Similarly, under predicted climate change scenarios, changes in environmental variables lead to a shortage in honey bee forage, which in turn feeds back negatively on pollination services and impacts honey yields (Delgado et al. 2012). Climate influences honey production through its effect on nectar availability for foraging honey bees. Nectar production in flowering plants is dependent on many complex factors such as flower size, age and condition of flowers, soil type, and time of day, but climate is one of the most important drivers (Crane 1975; Maurizio 1975; Phillips et al. 2018). Therefore, environmental fluctuations such as changes in rainfall and temperature that affect nectar availability also greatly influence honey production (Moffett and Parker 1953; Beerlink 1992; Langowska et al. 2017).

The Western Cape Province and the West Coast of South Africa is predicted to become warmer and drier under climate change models (Midgley et al. 2005). In 2018, an article was published in the South African Bee Journal in which many prominent beekeepers voiced their concerns about the low rainfall experienced in the Western Cape, with some also mentioning that certain honey crops are lost when sufficient rain does not fall at the right time of year (Ashley Cooper 2018). They had just entered their third year of very low rainfall, which was negatively affecting their bees and they contended that this was caused by very poor nectar secretion and a severe reduction in reliable bee forage. With these concerns from local beekeepers in mind, we wanted to ascertain the link between rainfall and honey production, which in turn is an indication of available forage and consequently affects honey bee colony conditions and pollination services.

The honey production records for selected apiary sites along the West Coast of South Africa were obtained for the 15-year period between 2002 and 2016, and corresponding rainfall data were obtained from a centrally located weather station on the West Coast via the South African Weather

Service. These data were used to test the relationship between annual honey production and rainfall (Supplementary Material). Initially the effect of rainfall in the current year compared to the two preceding years was assessed to ascertain whether there was a lag between rainfall and optimal nectar production. Since colony numbers are not static and honey production depends not only on resource availability but also on the number of honey producing units, the number of colonies was also included in the model. The number of hives producing honey, as well as the rainfall of the corresponding year, were the best predictors of honey production (Table S4). Since rainfall was found to be important in the current year, it was useful to discern whether the rain during the honey harvesting period or the period preceding the honey harvest was more predictive of honey yield. The rainfall in the six months preceding the honey production months (March to August) positively influenced the honey production of that year (Table S5). Annual honey production per beehive is thus correlated with annual rainfall (Figure 1), which will have the greatest impact if it falls in the winter months before the honey season starts. This is similar to the results found by Delgado et al. (2012), who showed that precipitation during the wettest month was positively correlated with honey yields.

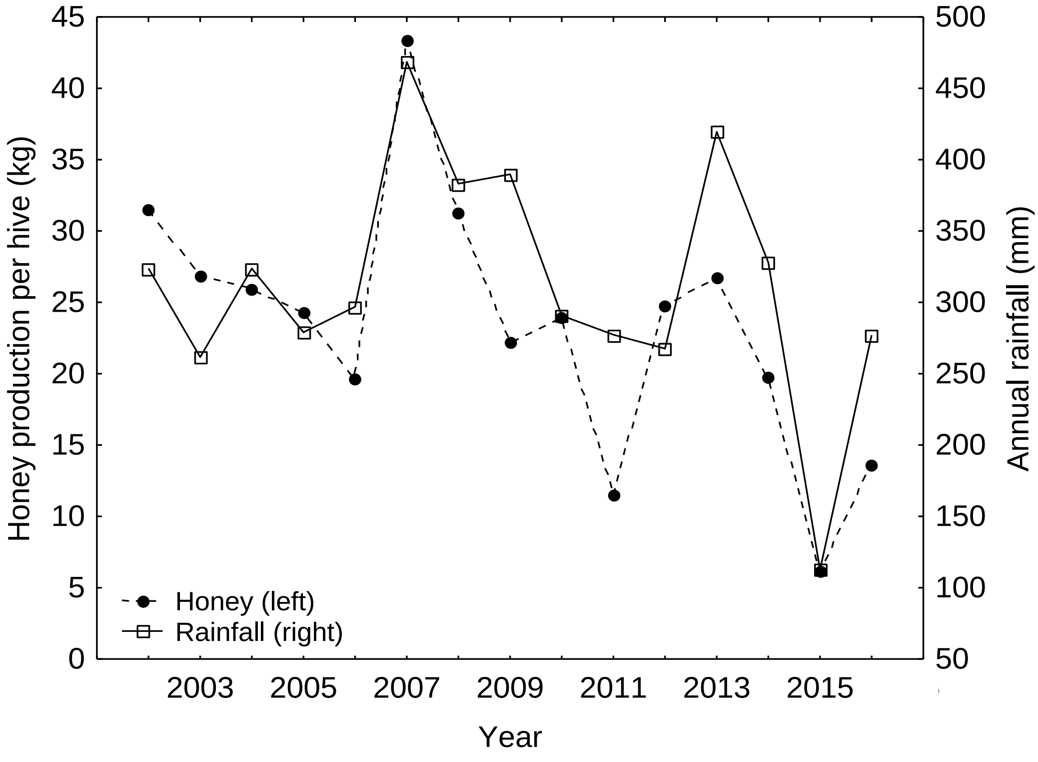

Figure 1: Honey production per beehive (kg) across selected apiary sites along the West Coast of South Africa, plotted against annual rainfall (mm) for the 15 years between 2002 and 2016.

From the 36 beehives managed for this study across the six apiary sites, only 23.9 kg of honey was harvested in 2015. This is exceptionally low compared to the 360 kg that was harvested in 2016 and the 211 kg harvested in 2017. From the analyses above, it is clear that the extremely low rainfall received in the area is responsible for the very low honey yields found in 2015 and the relatively low sample sizes of most monofloral honeys in subsequent harvest years. The results of this study are thus likely not representative of past trends in honey production along the West Coast under normal rainfall conditions, but it provides a glimpse into what can be expected in the future as this area will likely be faced with uncertain climate events like increasing droughts. Warmer and drier conditions on the West Coast could also increase extreme events such as fire frequencies that can also have an effect on honey production in the area (Midgley et al. 2005). For example, in Figure 1, the sudden drop in honey production seen in 2011 that does not align with rainfall was due to a wildfire occurring at one of the major honey producing sites included in the dataset.

Nevertheless, these harsh years were still able to produce monofloral varieties, in particular sandbos (*Aspalathus spinescens* Thunb. form A) that delivered the most samples (n=13) throughout this study and in the driest two years (2015 and 2017). This suggests that sandbos might be a valuable bee-plant for honey production in future drier years. In order to optimise honey production from this unique West Coast species and other monofloral varieties, specific areas for honey production must be identified where these plants occur in abundance. It was demonstrated that honey bees prefer and utilise similar resources across space and time (Chapter 2), which was also supported by previous findings of Johannsmeier (2001) that showed similar plant taxa to be important for honey bees in the area. Field-based information like this is important for optimising honey production in general, i.e. to identify and target specific sites where plant species preferred by honey bees occur. Future cultivation of bee-plants to produce unique monofloral varieties from indigenous fynbos vegetation should also be investigated, to follow suit with other natural, locally produced products from indigenous vegetation such as rooibos tea (*Aspalathus linearis* (Burm.f.) R.Dahlgren) and honey bush tea (*Cyclopia* spp.) with high economic value (Wilson 2005; Joubert et al. 2011). Cultivation of honey producing crops will also help address the current issue of honey bee forage availability, which is one of the biggest threats facing the beekeeping industry of South Africa. However, planting complete monocultures of these plants should be avoided. Other indigenous plants flowering in the area should be retained within close proximity to provide honey bees with the diverse diet they require, which is essential to bee health (Brodschneider and Crailsheim 2010).

Limitations and recommendations

Of the limitations already mentioned in each chapter, the main overarching issue was the limited sample sizes per botanical origin. This made it difficult to draw conclusions about the specific physicochemical properties and antibacterial activity that could potentially increase the marketability of these honeys and set them apart as market leaders. Regrettably, the sample size was constrained by the severe drought during this study period (Botai et al. 2017; Otto et al. 2018). More samples from each monofloral variety would have allowed a more definitive characterisation of each honey type. Many of the monoflorals in this study appeared to be very reliant on water availability, which begs the question whether more effort should go into characterising honeys that are sporadically harvested and are likely to become even more variable along the West Coast in the predicted changing climate (Midgley et al. 2005). It would be more prudent to invest in identifying drought-tolerant bee-plants that are affected less by the drying of the West Coast and produce good quality monofloral honey yields with potential antimicrobial activity.

Almost 70% of the fresh honey samples tested showed antibacterial activity above 10% phenol equivalence, thus were potentially therapeutically useful (Bischofberger et al. 2011; Irish et al. 2011). Here honeys were only tested against the gram-positive bacterium species, *Staphylococcus aureus* Rosenbach, although honey has been reported to have an inhibitory effect against ca. 60 species of gram-positive and gram-negative bacteria, including aerobes and anaerobes (Molan 1992; Brady et al. 1997; Blair and Carter 2005). Even though *S. aureus* is the most commonly used microorganism for the testing of honey antibacterial activity, the inclusion of only one bacteria species in our bioassays limited the assessment of the potential therapeutic properties of CFR honeys. A variety of gram-positive and gram-negative bacteria should be included in future studies to assess West Coast honeys more holistically.

Identifying the pollen grains trapped in honey is used to classify the geographic and botanical origin of honey (Von der Ohe et al. 2004). A challenge that is faced when quantifying the contribution of different plant species to the botanical origin of honeys, is that the relative pollen counts of all plant species might be an under- or over-representation of the actual nectar contribution to the honey (Bryant and Jones 2001; Rodopoulou et al. 2018). This issue of over- and under-represented pollen types is a major concern of any melissopalynological study in areas where pollen coefficient values are not available for bee-plant species. As Johannsmeier (2001) also pointed out, high foraging rates on plant species belonging to the Proteaceae family was observed – yet very little pollen from these species were identified in the honey samples (except one monofloral *Serruria* honey obtained from

Kersefontein 1). These observations would suggest that the nectar contribution to the honeys sampled should be much higher than what was indicated by the pollen counts and it is possible that monofloral samples produced from the protea family are simply undetected. This notion could potentially be investigated through caged honey bee experiments to obtain pollen coefficient (PC) values for local indigenous flora (Bryant and Jones 2001; discussed in Chapter 2). However, as the CFR is so diverse in plant species (~ 9000 species; Manning and Goldblatt 2012), this approach might pose logistical issues and due to resource limitations could probably only be used to obtain the PC values of a few focal bee-plant species.

Additionally, melissopalynology is a very laborious process and using a big pollen library for the identification of the botanical origin of honey is challenging. Here the focus was on a very small area of the West Coast, which was difficult in itself, and this questions the practicality of using this method over large areas of the CFR. Generating subset pollen libraries that spatially and temporally correlated the specific honey samples to field data, specifically to those plants that could potentially have contributed to the samples helped to make the library more user-friendly. Additional assistance in the form of DNA meta-barcoding analyses of the honey samples is a future direction that should be investigated. If the financial limitation of expensive genetic analyses can be overcome, this method could effectively be used in conjunction with pollen analyses. When a pollen library is compiled for a specific area, duplicate plant samples can be collected, and a concurrent DNA database generated for the respective species. With genetic analyses it will be possible to obtain a list of plant species that contribute to the honey based on their genetic differences, and thus subsequent melissopalynology analyses with a predetermined list of potential pollen types will be a lot easier. Using DNA methods will, however, still not resolve the issue of under- or over-representation of pollen from certain floral sources.

Over a three-year period, 66 honey samples from a 40 km stretch along the West Coast of South Africa were characterised based on their botanical, physicochemical and antibacterial qualities. If the aim is to characterise honeys across large expanses of the CFR in order to identify potential market leader honeys, a larger scale approach will be needed to make the process more efficient. Starting a citizen science project where the public can send in pollen samples and photos of the plants where they see honey bees foraging, for example, could help reduce the time that is needed to build a complete CFR pollen (and DNA) library by individual researchers. Citizen science is increasing in popularity worldwide and in areas such as biodiversity surveys, recruiting the public to take part in research projects is very valuable (Kobori et al. 2016). For example, The Global Biodiversity Information Facility, the world’s largest such repository, states that half of its billions of data points come from citizen sources (Irwin 2018). Similarly, collecting pollen grains from national botanical gardens where many identified plant species co-occur, could also help to streamline the database building process. The pollen library should be housed centrally so that it is easily accessible to the wider scientific community and researchers not necessarily working on honey only. A suggestion for this location could be with the South African National Biodiversity Institute (SANBI) at Kirstenbosch National Botanical gardens. It will also be important to include beekeepers in this broad scale honey characterisation approach, because they have the landscape-specific knowledge about which plant species honey bees use in their specific areas. If beekeepers can send in pollen samples of the plant species they believe to be the most important contributors to their honey samples and additionally donate honey samples from these harvests, it will be possible to verify the origin of the honeys and to conduct the subsequent physicochemical property analyses and antibacterial activity assays faster.

Value of this research for beekeeping in South Africa

In this study, a physical and electronic pollen library was generated – the first of its kind in South Africa. The thorough collection of pollen over the three years in the field allowed for most of the pollen types in the honey samples to be successfully identified, at least up to family level. Specific sizes of pollen grains in honey were also measured, which will help optimise the post-harvest / pre-packaging phase for Hurters Honey and beekeepers in the CFR in general. Information about the size of fynbos pollen grains will ensure that the correct honey filters can be used during honey packaging, to retain the majority of pollen grains for the production of raw honey. The electronic pollen pictures prepared during this study will be hosted on a Stellenbosch University website and will be made available to serve as a reference to future researchers and the broader scientific community.

This study was designed in conjunction with Hurters Honey (Langebaan, South Africa), the industry partner on this project. This honey producer is ideally situated within the West Coast region and monopolises the honey production within the area, yet they have not capitalised on the unique flora available and marketed any value-added products. The data obtained from investigating the botanical origin, the physicochemical properties and the antibacterial activity of selected West Coast honey was intended to provide Hurters Honey with marketing tools for promoting their products in the local and international honey markets. Unfortunately, due to the low sample sizes obtained for monofloral honeys during the study because of the drought, no definitive recommendations for value-added marketing could be provided. However, it was confirmed that the majority of honey samples from the West Coast adhere to local and international physicochemical standards. This should appease beekeepers that the South African regulations are adequate for assessing fynbos honeys. Overall, the

West Coast honey samples showed potentially therapeutically beneficial antibacterial activity to the wound bacterium, *S. aureus*. This study also identified potential drought-tolerant monoflorals that could be utilised for honey production as the West Coast is predicted to become warmer and drier in the future (Midgley et al. 2005). Obtaining and analysing more honey samples in subsequent years could facilitate the identification of additional plants species that may be of value to honey production in the area.

When more honey samples from across the CFR are obtained and characterised, this research will deliver information regarding the unique characteristics of West Coast honeys, which could earn it a higher price on both local and international markets. This will serve as a financial incentive to increase honey production and consequently lead to an increase in the number of managed honey bee colonies. This does not only mean more honey production and the production of value-added niche products, but also more resources to fulfil our agricultural pollination needs. Such an increase in managed honey bees could be imperative at a time when there is a threat of a global pollination crisis (van der Sluijs et al. 2016; Marshman et al. 2019). The increased price of honey and an increase in honey production will be a welcome boost to local beekeepers who are currently under severe pressure, especially from cheap honey imports.

Additionally, identifying important bee-plants in the area that could have possible economic benefits of producing a very marketable honey, will lead to more patches of natural vegetation being preserved by farmers. This may additionally lead to the conservation of many other species in the mosaic of agricultural land, but most importantly, it will increase the potential for honey production in the area. Knowledge of the specific plant species most valuable for honey production could help Hurters Honey identify new honey production sites based on the vegetation that already occurs there. This could lead to the conservation of more natural areas within an agricultural landscape and it might be prudent in considering the cultivation of more areas of the important bee-plants. Moreover, an increase in the demand for honeys produced from indigenous vegetation could also increase the need for a greater staff complement in both beekeeping and the honey production and packaging industry, ultimately creating more jobs and economically boosting local communities and the beekeeping sector of South Africa.

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Supplementary Material

Methods

Traceability of equipment

In response to recent discoveries of the honey bee diseases such as American Foulbrood (AFB) along the West Coast of South Africa, the following methods were implemented to prevent the possible spread of any diseases between apiary sites:

A colour was assigned to each site. This colour was painted on all equipment – including supers, super frames, gloves and hive tools to be used at a specific site – to ensure that no equipment would be moved between sites. Duplicate sets of gloves and hive tools were used, and each set of equipment remained on its specific site for the duration of the study. Super frames were marked with numbers corresponding to specific sites and specific hives, so that frames would not be moved between farms and always returned to their original hives and honey supers.

All the equipment used in this study was either brand new or freshly irradiated with gamma rays by HEPRO (High Energy Processing) in Cape Town. The irradiated supers and honey frames were sealed in plastic bags for transport to the storage room on Middelkraal. The bags were only opened briefly in order to mark the equipment as mentioned above. If any extra equipment such as hive lids, queen excluders, supers or frames were needed during the course of the experiment, only brand new or irradiated equipment were brought in and colour-marked according to the specific farm where it was required.

Before and after every fieldwork season, the brood boxes of all beehives were inspected for potential honey bee diseases. No notifiable diseases were detected at any of the apiary sites over the three-year study period.

Statistical analyses

The honey production records for selected apiary sites managed by beekeepers Anita and Heinrich Grunder along the West Coast of South Africa were obtained for the period between 2002 and 2016, and coinciding rainfall data were obtained from a centrally located weather station via the South African Weather Service. To test the relationship between honey production and different annual rainfall variables, two Generalized Linear Models (GLZ) with Gaussian distributions were run using the MASS package in RStudio version 1.2.1335 (RStudio Inc., USA).

In the first model, total honey production per year was the dependent variable tested against three predictor variables: the total number of beehives across the apiary sites, the corresponding year's annual rainfall and the rainfall in the two preceding years (Rain, Rain-1 and Rain-2; Table S4). In the second model, the total number of beehives across the apiary sites, rainfall in the 6 months preceding honey production (March to August) and rainfall during honey production months (September to February) were predictor variables tested against total honey production per year as the dependent variable (Table S5).

Figures

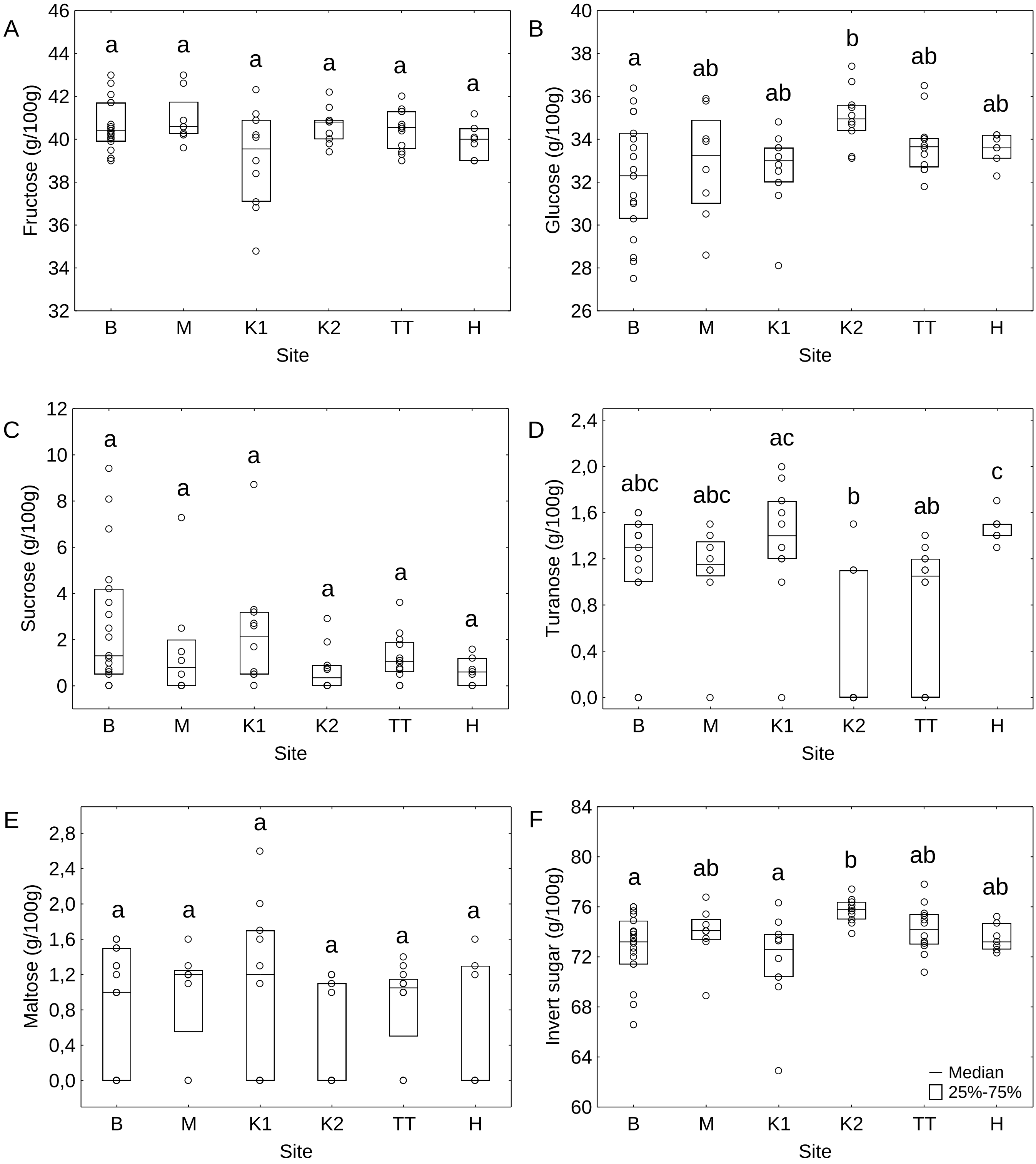

Figure S1: A comparison of the sugar composition between honeys harvested at different apiary sites along the West Coast. The different panels show: Fructose content (A), glucose content (B), sucrose content (C), turanose content (D), maltose content (E) and the total invert sugars (F) in grams per 100 g of honey. Significant differences based on Kruskal-Wallis tests with multiple comparisons of the mean ranks of all groups are indicated with different letters ($p < 0.05$). Boplaas = B (n = 17), Middelkraal = M (n = 7), Kersefontein 1 = K1 (n = 10), Kersefontein 2 = K2 (n = 10), Thali Thali = TT (n = 12) and Hopefield = H (n = 7).

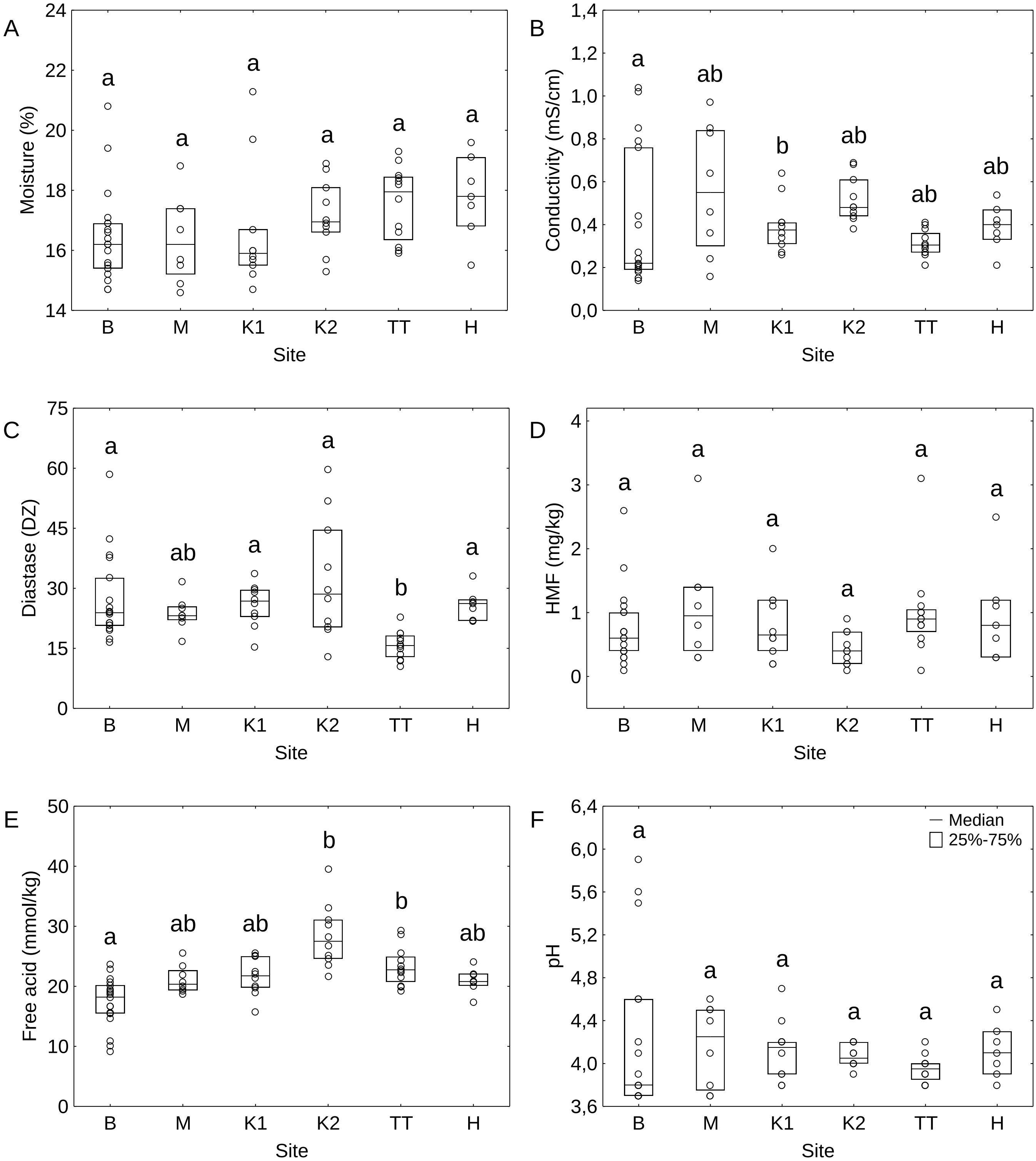

Figure S2: A comparison of physicochemical properties between honeys harvested at different apiary sites along the West Coast. The different panels show: Moisture content (A), electrical conductivity (B), diastase activity (C), hydroxymethylfurfural (HMF) content (D), free acid content (E) and pH (F). Significant differences based on Kruskal-Wallis tests with multiple comparisons of the mean ranks of all groups are indicated with different letters ($p < 0.05$). Boplaas = B (n = 17), Middelkraal = M (n = 7), Kersefontein 1 = K1 (n = 10), Kersefontein 2 = K2 (n = 10), Thali Thali = TT (n = 12) and Hopefield = H (n = 7).

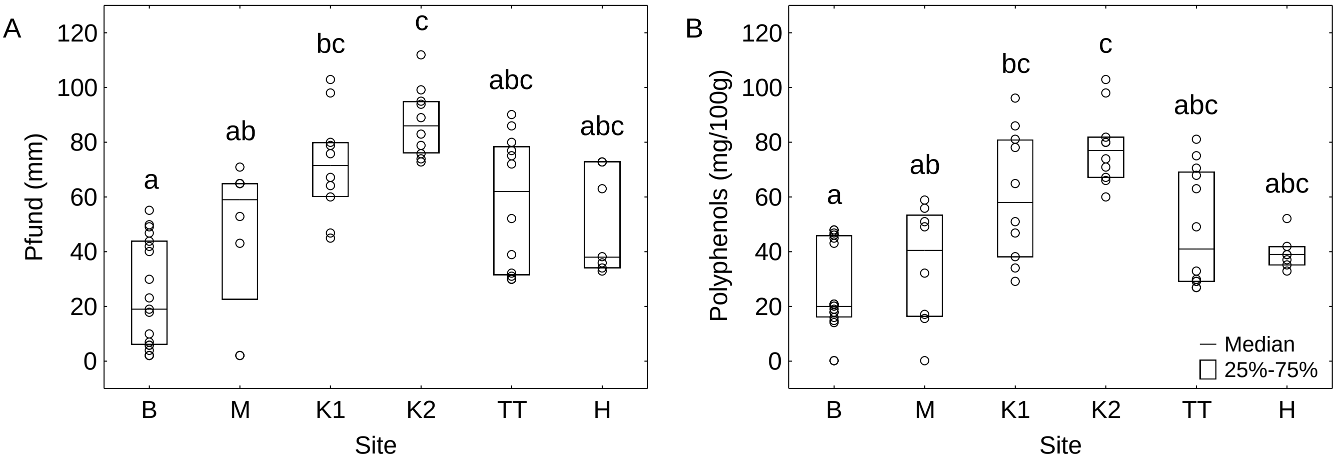

Figure S3: A comparison of colour (panel A) and polyphenol content (panel B) between honeys harvested at different apiary sites along the West Coast. For colour and polyphenol content significant differences are indicated with different letters ($p < 0.05$), based on Kruskal-Wallis tests with multiple comparisons of the mean ranks of all groups. Boplaas = B (n = 17), Middelkraal = M (n = 7), Kersefontein 1 = K1 (n = 10), Kersefontein 2 = K2 (n = 10), Thali Thali = TT (n = 12) and Hopefield = H (n = 7).

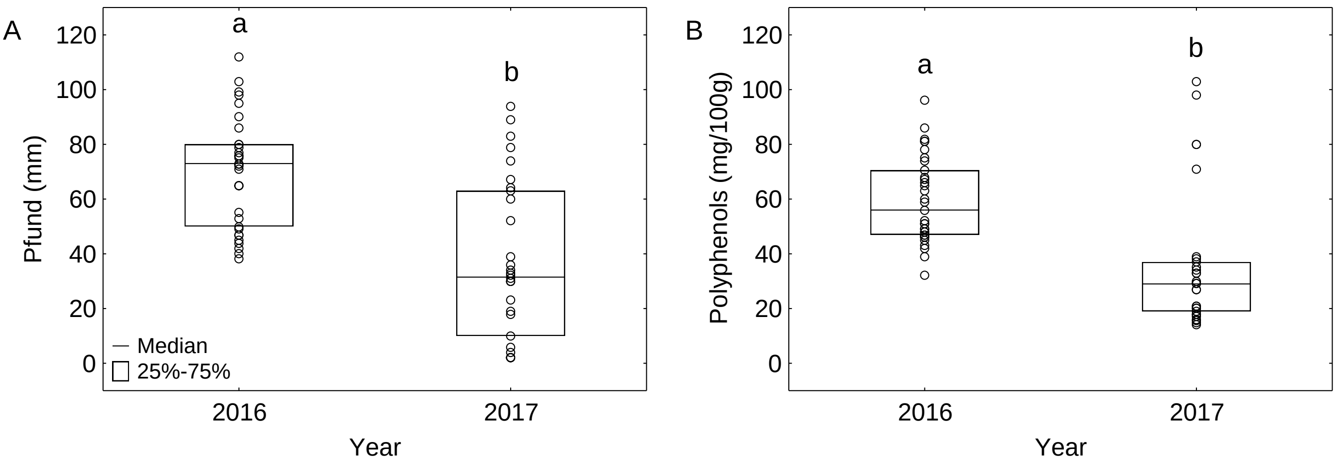

Figure S4: A comparison of colour (panel A) and polyphenol content (panel B) between honeys harvested along the West Coast in 2016 (n = 33) and 2017 (n = 30), respectively. For colour and polyphenol content significant differences are indicated with different letters ($p < 0.05$), based on Mann-Whitney U tests.

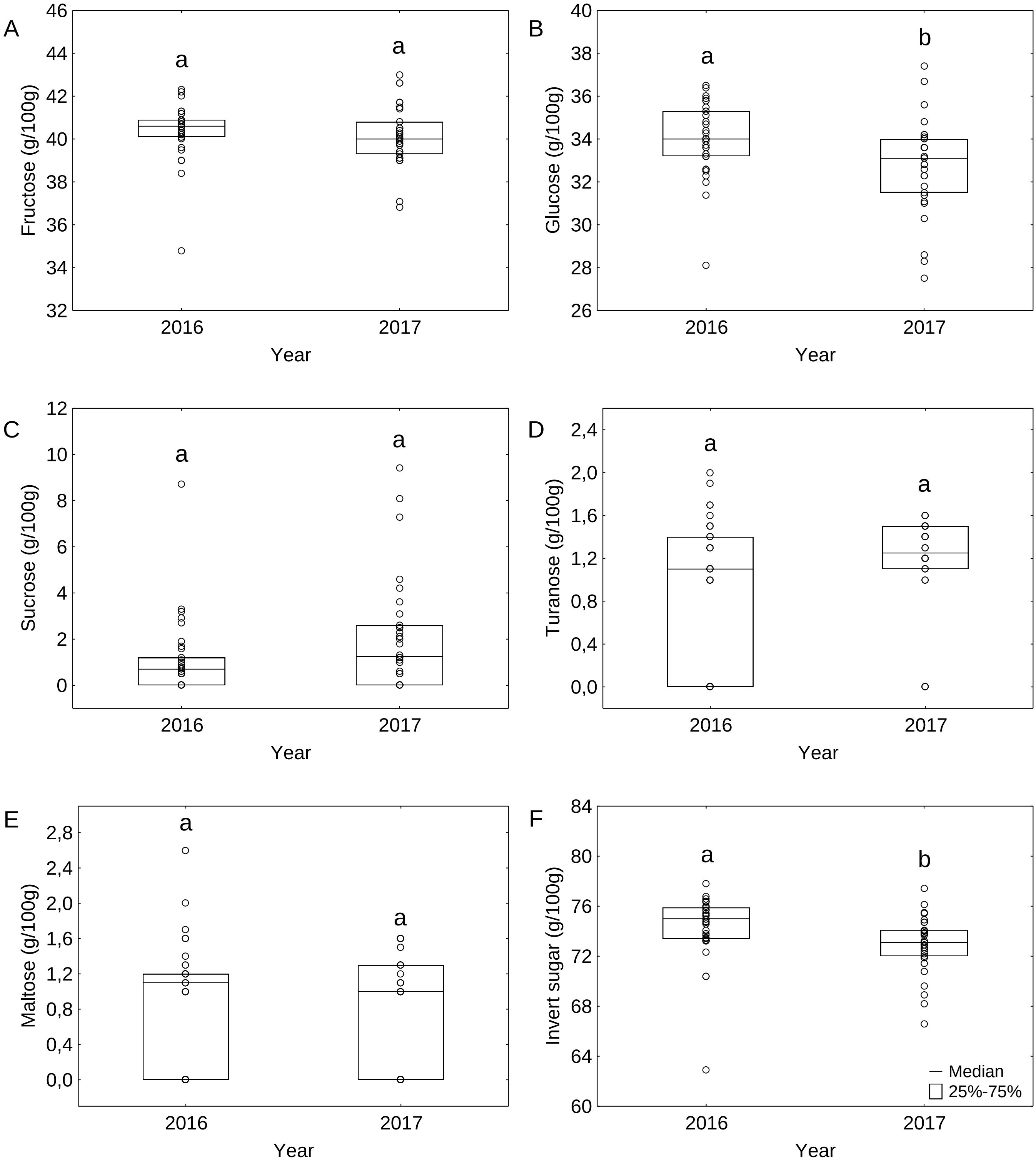

Figure S5: A comparison of the sugar composition between honeys harvested along the West Coast in 2016 (n = 33) and 2017 (n = 30), respectively. The different panels show: Fructose content (A), glucose content (B), sucrose content (C), turanose content (D), maltose content (E) and the total invert sugars (F) in grams per 100 g of honey. Significant differences based on Mann-Whitney U tests are indicated with different letters ($p < 0.05$).

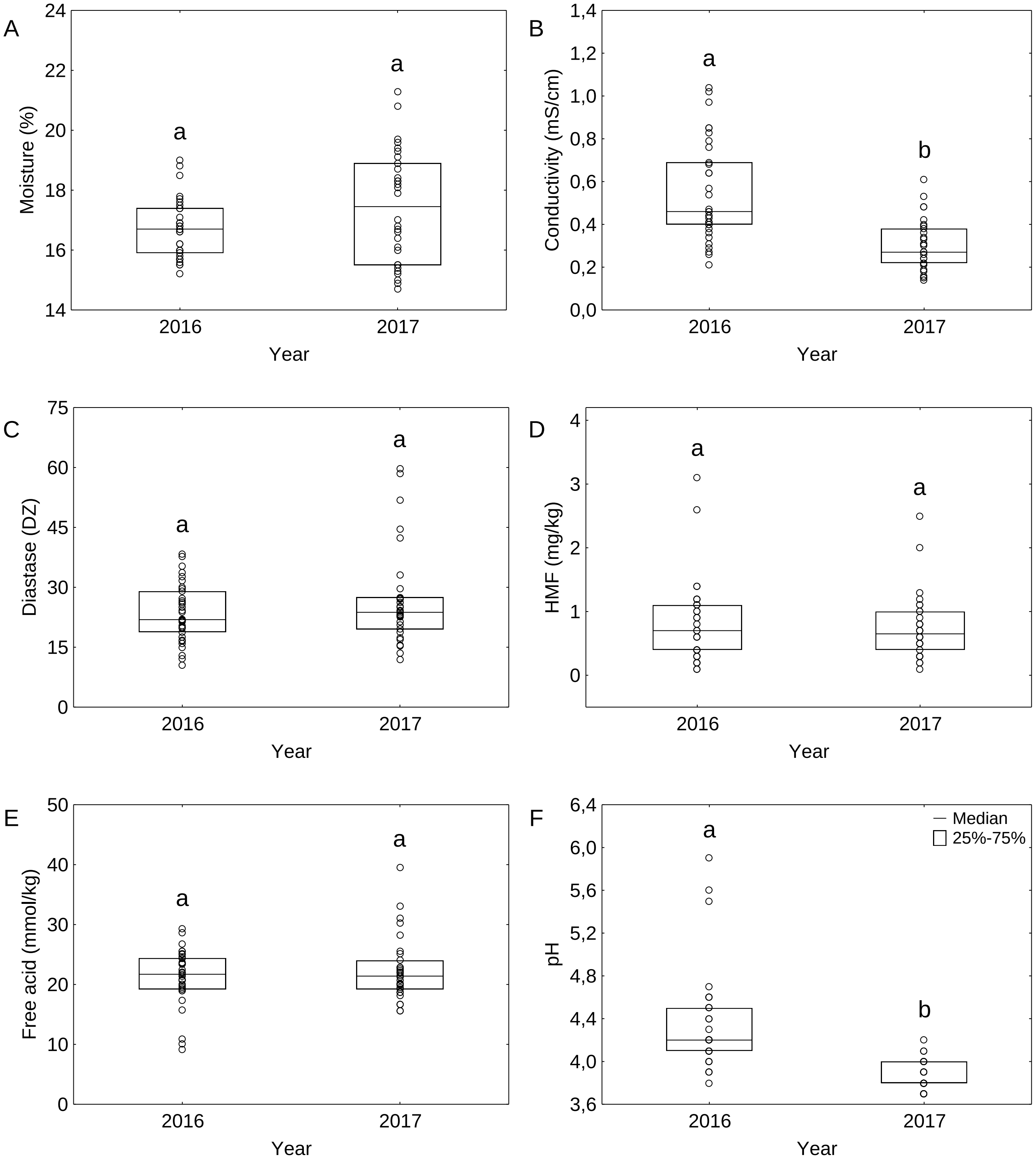

Figure S6: A comparison of physicochemical properties between honeys harvested along the West Coast in 2016 (n = 33) and 2017 (n = 30), respectively. The different panels show: Moisture content (A), electrical conductivity (B), diastase activity (C), hydroxymethylfurfural (HMF) content (D), free acid content (E) and pH (F). Significant differences based on Mann-Whitney U tests are indicated with different letters ($p < 0.05$).

Tables

Table S1: The 23 plant species observed to be attractive to honey bees that were surveyed in each quadrat at the six apiary sites along the West Coast to assess the availability of bee-plants (n = 10 quadrats per apiary site).

Family	**Species**
Aizoaceae	*Carpobrotus acinaciformis* (L.) L.Bolus
Aizoaceae	*Conicosia pugioniformis* (L.) N.E.Br.
Anacardiaceae	*Searsia laevigata* (L.) F.A.Barkley
Ebenaceae	*Diospyros austro-africana* De Winter var. *rugosa* (E.Mey. ex A.DC.) De Winter
Ebenaceae	*Euclea racemosa* Murray
Fabaceae	*Aspalathus hispida* Thunb.
Fabaceae	*Aspalathus spinescens* Thunb. (form A)
Fabaceae	*Aspalathus spinescens* (form B)
Fabaceae	*Aspalathus stricticlada* (R.Dahkgren) R.Dahlgren
Fabaceae	*Calobota angustifolia* (E.Mey.) Boatwr. & B.-E.van Wyk
Fabaceae	*Otholobium venustum* (Eckl. & Zeyh.) C.H.Stirt.
Fabaceae	*Wiborgia fusca* Thunb.
Fabaceae	*Wiborgia mucronata* (L.f.) Druce
Lamiaceae	*Salvia africana-caerulea* L.
Lamiaceae	*Salvia lanceolata* Lam.
Malvaceae	*Hermannia scabra* Cav.
Malvaceae	*Hermannia trifurca* L.
Polygalaceae	*Muraltia scoparia* (Eckl. & Zeyh.) Levyns
Polygalaceae	*Muraltia spinosa* (L.) F.Forest & J.C.Manning
Proteaceae	*Leucospermum hypophyllocarpodendron* (L.) Druce
Proteaceae	*Leucospermum rodolentum* (Salisb. ex Knight) Rourke
Proteaceae	*Serruria fucifolia* Salisb. ex Knight
Rutaceae	*Agathosma bisulca* (Thunb.) Bartl. & H.L.Wendl.

Table S2: Measurements of physicochemical properties of South African honeys, selected from Anderson and Perold (1964). The unit of measurement as well as the official quality standards for blossom honey are indicated in bold next to each physicochemical parameter. For the parameters where no official standard value is provided, general ranges are indicated as obtained from published literature (not in bold). The mean, minimum and maximum values for each physicochemical parameter measured by Anderson and Perold (1964) is given and indicated in bold if it falls outside the permitted range of either Codex (2001) or South African Department of Agriculture (2000) standards.

	Standards and Ranges				**South African honeys**		
	Unit	**Codex**	**DOA**	**n**	**Mean**	**Min**	**Max**
Fructose (F)	%	27.2 - 44.3 [1]		66	35.50	22.98	40.15
Glucose (G)	%	22.0 - 40.7 [1]		66	31.54	22.32	39.43
Invert sugars (F+G)	%	**> 60**	**> 65**	66	67.04	**45.30**	79.58
Sucrose	%	**< 5**	**< 5**	66	0.54	0.00	**6.24**
Maltose*	%	2.7 - 16.0 [1]		66	5.38	2.07	10.02
Moisture	%	**< 20**	**< 20**	66	16.23	14.12	18.80
Ash	%		**< 0.6**	66	0.33	0.03	**0.94**
pH	pH	3.4 - 6.1 [1]		66		3.36	4.62
Pfund	mm	0 - 150 [2]		66		64	131.4
Nitrogen	%	0.00 - 0.13 [1]		66	0.04	0.02	0.13
Copper	ppm	0.14 - 1.04 [3]		19	0.55	0.25	0.83
Potassium	ppm	100 - 4733 [3]		19	1105	141	2945
Sodium	ppm	6 - 400 [3]		19	154	34	806
Calcium	ppm	23 - 266 [3]		19	106	36	164
Phosphorous	ppm	23 - 58 [3]		17	37.20	19.93	58.43
Iron	ppm	1.20 - 33.50 [3]		17	5.68	2.65	8.42
Manganese	ppm	0.17 - 9.53 [3]		17	2.13	0.06	6.15

1. White et al. 1962; 2. Hannah Instruments, USA; 3. White 1975; *measurement includes all reducing disaccharides

Table S3: Pfund values of the different honey colour categories

Honey colour	**Pfund (mm)**
Water white	0 - 8
Extra white	8 - 16.5
White	16.5 - 34
Extra light amber	34 - 50
Light amber	50 - 85
Amber	85 - 114
Dark amber	> 114

Table S4: Generalized Linear Model output testing total annual honey production at selected West Coast apiary sites against the predictor variables: number of hives honey was harvested from, the rainfall in the corresponding year to the honey production (Rain), and the rain in the two preceding years (Rain-1 and Rain-2). Bold p-values indicate significance ($p < 0.05$).

	Estimate	**Std. error**	**t-value**	**p-value**
Intercept	-3921.66	3614.95	-1.09	0.304
Number of hives	15.58	3.52	4.42	**0.001**
Rain	24.15	5.53	4.37	**0.001**
Rain-1	4.44	5.12	0.87	0.406
Rain-2	-9.53	6.82	-1.40	0.192

Table S5: Generalized Linear Model output testing total annual honey production at selected West Coast apiary sites against the predictor variables: number of hives honey was harvested from, the rainfall in the corresponding year occurring in the 6 months before honey production (Rain before) and the rainfall occurring during the 6 honey production months (Rain during). Bold p-values indicate significance ($p < 0.05$).

	Estimate	**Std. error**	**t-value**	**p-value**
Intercept	-5699.80	2274.30	-2.51	**0.029**
Number of hives	14.25	3.93	3.63	**0.004**
Rain before	24.99	8.45	2.96	**0.013**
Rain during	28.45	18.83	1.51	0.159

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